Host-Parasite Relations of the Fungus *Dothidella ulei* P. Henn on the Hevea Rubber Tree

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INTRODUCTION[1]

Dothidella ulei P. Henn. causes a destructive disease of the foliage of the Para rubber tree, Hevea brasiliensis Muell. Arg., and is known as the South American leaf blight disease. It has been the primary cause of the failure or near ruin of many plantations in the Guinas and the Ford plantations at Fordlandia, Belterra, Belem, Brazil. Klippert (12) stated that leaf blight was a major factor in preventing the establishment of a plantation rubber industry in the Americas.

Kuyper (14), Stahel (25), Weir (30), and Rands (21) have stated that the only susceptible parts of the trees to infection by Dothidella ulei were rapidly growing leaves, shoots, fruits, and flowers.

Stahel (25) conducted a comprehensive study of the disease, and concluded that leaves up to 2 weeks old were highly susceptible, and that with continued growth they gradually became resistant to fungus attack.

Hilton (10) stated that the symptom expression always varied with the age of the leaf attacked, and this was particularly marked in Hevea, in which the leaves pass through a series of well defined stages.

Langford (15) stated that leaves are at their maximum susceptibility from the time of emergence until they are 7 to 10 days old. Inoculations on 2-week-old leaves of susceptible clones induced

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[1]This grant-in-aid fellowship was sponsored by the Firestone Tire and Rubber Co., Akron, Ohio.

numerous non-sporulating lesions after 15 to 20 days.

A system for the classification of Hevea clones for resistance to D. ulei was proposed by Langford (15), based on the tolerance of the trees to the disease and the partial to complete inhibition of fungus sporulation.

Blazquez and Owen (2) reported that fungus growth was favored by small concentrations of i-inositol and quebrachitol (2-mono-methyl ether of l-inositol), but that at high concentrations of both i-inositol and quebrachitol growth of the fungus was inhibited. They stated that quebrachitol was present in the latex of species of Hevea in concentrations of 0.5 to 2.0 per cent, and that it may be present in smaller amounts in the latex of young leaves. They also stated that this may be the reason why young Hevea leaves are susceptible to infection to this fungus whereas older leaves are not infected or only slightly infected.

The purpose of this work was to make comparative studies of the host-parasite relationship of this disease using Hevea clones of different degrees of resistance. Histological studies were made to determine, if possible, what host reactions took place within the tissues of diseased susceptible clones as compared to diseased resistant clones. Biochemical investigations including chromatographic, nutritional, and microchemical experiments were also conducted in an attempt to find out whether or not certain compounds which might be antagonistic to development of D. ulei existed in greater concentrations in clones of a higher degree of resistance than in the highly susceptible clones.

DISEASE DEVELOPMENT ON LEAVES OF DIFFERENT STAGES OF GROWTH

Four clonal selections of Hevea brasiliensis Muell. Arg. which possessed varying degrees of susceptibility and resistance were obtained from the United States Department of Agriculture, Plant Introduction Gardens, at Coconut Grove, Florida.[1] A number of Tjir 1 x Tjir 16 seedlings were budded with buds from the 4 clonal selections. After the buds had taken, the trees were topped, packed and shipped to Gainesville, where they were planted in beds inside a greenhouse. Attempts were made to control the moisture and temperature within the greenhouse as near as possible to the optimum for disease development. Budwood from each clone was brought to Gainesville and budded to seedlings growing under greenhouse conditions to insure representative samples of each clone (Table 1).

Good growth of the seedlings and budded clones was maintained by adding iron, magnesium, manganese, zinc, nitrogen, and potassium in dilute solutions once a week.

The solutions were prepared following the recommendations of Professor Seton N. Edson, of the Soils Department, University of Florida. The amounts of chemicals added as fertilizers are shown in Table 2.

The fastest growing trees were topped to keep them at workable

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[1]Courtesy of Mr. Jeffrey B. Shrum, Horticulturist, United States Department of Agriculture, Plant Introduction Gardens, Coconut Grove, Florida.

TABLE 1

DESIGNATION OF CLONAL SELECTIONS SHOWING PARENTAGE AND RESISTANCE RATING

Clone	Parentage	Rating
Liberian	Tjir 1 x Tjir 16	10 ✓✓✓✓
IAN 45-873	PB-86 x FA-1717	8 ✓✓✓
FX 232	F 351 x PB-86	5 ✓✓
FX 2831	F 4542 x Tjir 1	3 0
P-122	H. brasiliensis (Iberia, Peru)	2 0

TABLE 2

AMOUNTS OF CHEMICALS USED AS FERTILIZERS IN STOCK AND WATERING SOLUTIONS

Elements	Stock solution g of element in 1 liter of water	ml Stock solution in 3 gal of water	Net amount of element in lbs/acre
$ZnSO_4 \cdot 7H_2O$	24.0	60	20
$NaNO_3$	98.1	30	10
KNO_3	45.9	60	20
$MnSO_4 \cdot H_2O$	32.4	75	25
$MgSO_4 \cdot 7H_2O$	160.5	300	100
Fe^{+++} as Sequestrin	1.3	3	2

heights, and to cause them to produce more foliage. Shading of the trees was provided by a netting at a height of 10 feet. Netting was also hung from the sides of the greenhouse, both to prevent gusts of wind from scattering the conidia and to increase the humidity. Flushes of growth were produced continuously by most trees. During the months of May until early October, when the minimum temperatures did not fall below 65° F. the greenhouse vents were left open.

During the colder months of the year the greenhouse vents were closed, and steam heat maintained a temperature of about 70° F. The dryness of the atmosphere under this type of heating was prevented by blowing air with an electric fan placed behind a mist sprayer in the upper section of the greenhouse.

Leaf Stage Designation

Six arbitrary designations were set up to facilitate leaf age classification depending upon growth characteristics of the Hevea leaves.

Stage I Leaves

In the earliest leaf differentiation a flush of leaves is composed of 3 sets of leaves and each leaf is composed in turn of 3 leaflets. The leaflets are folded dorsally and point upward. The petrioles are folded upward close to the growing point (Figure 1).

Stage II Leaves

Stage II is characterized by the centripetally bending leaves, eventually the tips are pointed straight down, and remain folded. The

Figure 1. Stages of development. (1) Stage I, (2) Stage II, (3) Stage III, (4) Stage IV, (5) Stage V, (6) Stage VI.

leaflets are very firm, have a reddish color, and are less than 3/4 of an inch in length. Some of the younger leaves of the flush may still be in Stage I. The petioles are separated and are pointing upward, away from the growing point at an angle of 30°. Dijkman (5) called this period stage "A" (Figure 1).

Stage III Leaves

Leaves are in this stage when pointing straight down, larger than 3/4 of an inch in length, firm, very shiny, rich in anthocyanin pigment, of a maroon color, part way open, and with the 3 leaflets hanging with their lower surfaces tightly pressed against the lower surfaces of the other leaflets, the petioles being bent 60° away from the growing point (Figure 1). (Dijkman's stage "B")

Stage IV Leaves

The leaves unfolded and hung vertically with their lower surfaces lightly pressed against the lower surfaces of the other leaflets. The reddish color of the leaflets changed to opaque olive green, and the petioles are separated to about an angle of 60° from the growing point (Figure 1). (Dijkman's stage "C")

Stage V Leaves

The expanding light-green leaves lose their olive-green color, and the petioles are separated from the growing point at an angle of 70° (Figure 1). (Dijkman's stage "D")

Stage VI Leaves

The fully extended light-green leaves change to dark green

with firm laminae, and petioles are separating from the growing point at an angle of 70° or more (Figure 1).

An experiment was designed to determine the effect of leaf age on the variation of symptoms and disease development on susceptible clonal selections.

Leaves of susceptible selections were inoculated simultaneously at various stages of development.

At suitable intervals after inoculation, data were taken on the appearance and development of infection on the individual leaves.

Macroscopic Observations on Living Leaves

Stage I. Heavily inoculated leaves produced a black exudate at the points of infection, which remained on the leaflets until they blackened, shriveled, and fell off. If only a few spores were placed on the leaves, the black exudate remained, adhered to the leaves, distorting their shape. Generally severely infected leaves showed extreme twisting and curling before shriveling and dropping off.

Stage II. Inoculated leaves showed minute raised areas after 3 days. Within a week they began to curl, and the fungus sporulated abundantly. The reddish tinge of the normal leaves changed to dark olive green as spores were produced on the under surface of the unfolding leaves. They blackened and shriveled, hung onto the petioles for less than a day and fell off, leaving the petioles still attached to the shoot or to the stem of a tree (Figure 2).

Stage III. Inoculated leaves larger than 3/4 of an inch, maroon in color and very shiny, showed 2 types of symptoms, a

Figure 2. Stages of leaf development 2 months and 3 weeks after inoculation. (2) Stage II, (3) Stage III, (4) Stage IV, (5) Stage V.

translucent condition previously described by other workers (14,15, 21,27,30) and a humid condition. In the translucent condition the lesions were clearly delimited by a marginal brownish-black line, which sometimes was very pronounced. The tissues within the border were very shiny and translucent. The green color of the leaf was much darker within the lesion, and in most cases was only near the marginal black line, but in others it was scattered throughout the lesion. Some small maroon areas were readily seen, since they were not masked by the opaque normal green color. The lesions were limited by the secondary veins. Generally, the lesions had numerous raised areas surrounding them which were lighter green than the normal tissues; however, these raised areas were not translucent. The translucent lesions were flat at first, with no visible raised areas, but as the disease progressed the raised areas became visible and more abundant around the limiting black margin of the lesion. The color of the raised areas gradually changed to a lighter green giving the translucent lesions a halo effect (Figure 3). The centers of the translucent lesions gradually became dark, and after 2 to 3 days changed to olive in color, and a velvety appearance developed due to the presence of spores. The raised marginal areas changed from a light-green color to olive, and spore development produced the velvety appearance as previously noted (Figure 4). Any leaf movement resulted in the release of a small cloud of spores from the under surface of the leaf.

Humid Condition.--The second type of symptom was observed only under 100 per cent relative humidity. It was similar to the raised areas surrounding a translucent lesion, except that the only raised

Figure 3. Translucent lesion with the halo effect of the lighter green raised areas.

Figure 4. Stages of leaf development one week after inoculation showing the velvety appearance of sporulating lesions. (3) Stage III, (4) Stage IV, (5) Stage V, (6) Stage VI.

areas were visible, without any translucent parts or centers of the lesions. Sporulation occurred in the same manner as previously described for the raised areas of the translucent lesions.

Macroscopic symptoms on inoculated leaves were of two types. The first and most common symptom was the gradual changing of the centers of olive lesions to dark green and ultimately to charcoal black. This black lesion was stromatic in nature and was surrounded by various distinct, erumpent, black dot-like stromatic structures, or spermogonia. There was a small yellowish circular area between the small spermogonia and the large black stromatic mass in the center of the lesion. The second type of symptom was a gradual change in color, from the outer perimeters of the raised areas surrounding the black limiting line of the translucent lesion toward the center of the lesion and including the raised areas. There was a definite dying of this inner marginal tissue, which became light yellow and then orange colored. The olive velvety mass formed on the translucent lesion gradually became black and stromatic, with few erumpent dot-like spermogonia in the center of the lesion. In some lesions there was a black necrotic line around the ring of dried tissue, while in others this black line was absent. On both types of lesions, masses of spores were produced on the top surfaces of the leaves from stromatic masses in the centers of the lesions.

Stage IV. Inoculated leaves showed small raised areas within 10 days after inoculation. These raised areas became rapidly discolored, and after 5 days black stromatic structures were present in their centers. On the lower surface of the leaf, masses of conidia

appeared directly opposite the stromatic structures on the upper surface. The number of lesions developed after inoculation was much less than the number appearing after inoculations at earlier stages of leaf development. The fungus sporulated moderately and formed few coalesced stromatic structures (Figure 5).

Stage V. Inoculated leaves showed symptoms similar to those observed in Stage IV.

Stage VI. Inoculated leaves produced very few lesions, and although they were similar to those developing on Stages IV and V they formed few erumpent black spermogonia and did not produce spores.

Inoculated leaves of greater maturity produced no symptoms although the dark olive-green masses of spores remained readily visible on the leaf surfaces.

Three types of symptoms were previously observed on the upper surface of leaves inoculated at Stages III and IV. The first type showed lesions with a black stromatic, erumpent, doughnut-like structure which may or may not have been surrounded by a light-green halo-like border. The doughnut-like stromatic structures were 1 to 3 mm wide, with dead and dry tissues ranging from light green to white in color in the center. The centers of the lesions fell out, and the characteristic "shot hole" described by Rands (21) was produced. In the second type the lesions had marked black necrotic margins surrounded by dead tissue and the black, erumpent, doughnut-shaped stromatic structure inside of the ring of dead tissues. There was a slightly green discolored area surrounding the entire lesion. In the third type of symptoms, no stromatic doughnut-shaped structure was

Figure 5. Stages of leaf development 2 months and 3 weeks after inoculation. (4) Stage IV, (5) Stage V, (6) Stage VI.

formed within the lesion, but rather a few black spermogonia were scattered throughout the lesion's dead and dry tissue inside a small light-brown necrotic marginal line. On the lower surfaces of the leaves, only flat stromatic tissue was observed directly underneath the spermogonia and the stromatic structures.

Inoculated leaves at Stage VI developed stromatic masses after 10 or more days which grew very slowly on the upper surface of the leaves. Within 1 month after inoculation black dot-like structures appeared on the upper surface of the leaves forming a ring, the center of which dried and either remained on the leaflet or fell away (Figure 5).

Leaf petioles, leaf veins, and growing shoots all gave the same type of symptoms at the various ages of the tissues.

Petioles and main veins of Stage I leaves produced the same type of black exudate as the laminae when heavily inoculated. The petioles blackened before being shed. If not heavily inoculated they often swelled, giving the leaves and growing shoot a twisted appearance. As the disease advanced these swellings cracked and sporulated heavy before turning black, shriveling and falling away.

Stage II petioles gave the same type of symptoms as the lightly inoculated Stage I petioles; however, as the age and size of the leaves increased, the swellings appeared as long streaks of sporulating cankers. These cankers acquired a woody appearance with age. The petioles of the Stage IV leaves and the lower parts of the growing shoots showed symptoms after inoculation.

Kuyper (14) reported that the swelling of the petioles was

due to the phloem tissue hypertrophy, particularly of the phloem parenchyma cells. Weir (30) stated that cankered stems bearing perithecia and terminating in well-developed young leaf clusters were frequently observed. Cankers were found on older stems, but the perithecial stroma had deteriorated and no ascospores were found.

Rands (21) was of the opinion in 1924 that within such a variable species as Hevea brasiliensis substantial differences in susceptibility occurred, and he reported that in every plantation attacked by the disease a few trees had remained healthy, made excellent growth and produced large canopies of dark-green foliage. He considered these trees to be resistant to the disease.

Langford (15) determined that resistance to the disease was exhibited in either or both of 2 forms: (1) tolerance of the disease by resistance to leaf damage and defoliation, and (2) partial or complete inhibition of the fungus. He proposed a system of classification of Hevea clones for resistance or susceptibility based on the extent of damage to leaves and the amount of fungus sporulation (Table 3). The classes he set up were for damage resulting to plants that were subject to heavy inoculation, and growing under conditions extremely favorable for disease development.

Heavily inoculated leaves of the resistant clone IAN 45-873 while at Stage I produced the same black exudate as highly susceptible leaves. Infected petioles also produced the black exudate. Lightly inoculated leaves at this stage produced a black exudate, but were not shed and became distorted and misshaped. The leaves were more distorted in areas where the black exudate was produced. Sparse

TABLE 3

CLASSES OF RESISTANCE OF THE HEVEA RUBBER TREE TO THE FUNGUS DOTHIDELLA ULEI P. HENN
Proposed by Langford[1]

1. Immune:[2] No evidence of infection.
2. Almost immune: Yellow flecks; no apparent damage.
3. Very highly resistant: Small necrotic lesions causing very slight damage.
4. Highly resistant: Necrotic lesions causing slight damage.
5. Resistant: Necrotic lesions causing distinct damage; leaves reduced in size and sometimes deformed.
6. Moderately resistant: Necrotic lesions causing conspicuous damage; leaves dwarfed and deformed or ragged.
7. Moderately susceptible: Defoliation ranging up to 50 per cent.
8. Susceptible: Severe to complete defoliation--more than 50 per cent.
9. Highly susceptible: Plant stem killed back. (New shoots may arise from secondary buds.)
10. Very highly susceptible: Plant killed by repeated defoliations.

The five symbols designating degree of sporulation are as follows:

0 = No sporulation.

\- = Very sparse sporulation: A maximum of two or three weekly sporulating leaf or petrioles lesions on the most diseased flush of leaves.

-- = Sparse sporulation: A few weakly sporulating lesions on the most diseased flush of leaves.

[1]Classes set up for damage under severe infection (15).

[2]The term Immune is used throughout this investigation following Langford (15).

TABLE 3--Continued

--- = Moderate sporulation:	Very noticeable sporulation on the most diseased flush of leaves, usually some on several other flushes.
---- = Heavy sporulation:	Conspicuous sporulation on the most diseased flush of leaves; usually some on most other flushes.

sporulation was observed on the lower surface of the leaves. After 3 weeks the leaves turned yellow and were shed. Leaves of Stages II and III gave similar symptoms when heavily inoculated on the lower surface. Lesions produced at these 2 leaf stages were similar to inoculations carried out on highly susceptible Stage IV leaves. On the upper surfaces of the leaves stromatic structures were observed with light-green areas and a light-brown center around raised lesions. Sporulation was observed on the lower surfaces of the leaflets opposite these brown centers, and as the disease progressed these areas became necrotic, ceased sporulating and the leaflets acquired a crinkled and stunted appearance. Some lesions, very minute in size with light-brown necrotic areas, which later turned into streak-like lesions, were observed on petioles and midribs of the leaves (Figure 6).

Leaves inoculated at Stage IV developed 2 kinds of lesions on the lower surface. Lesions of the first type were raised, light-green areas and yellow centers. Other lesions observed had irregular necrotic areas with large centers of dead tissue. Some sporulation was observed on this clone near young lesions on the laminae. Some lesions were found on the secondary veins and veinlets. No stromatic structures were observed on the veins or veinlets of the lower surface of the leaflets. On the upper surface of the leaflet some lesions showed only light-green depressed areas, while others had necrotic borders and large centers of dead, dry tissue. Inoculated leaves at older stages produced no visible symptoms and remained normal. Heavily inoculated leaves of Stage I of resistant clone FX 232 produced the black exudate observed on other resistant and susceptible clones. Leaves

Figure 6. Stage III leaflets of resistant clones 3 days after inoculation showing translucent lesions. (2) Clone IAN 45-873, (3) Clone FX 232, (4) Clone FX 2831, (5) Clone P-122 showing pinpoint centers of the lesions.

with black exudate adhering to their surfaces grew abnormally and were twisted and wrinkled. The more conspicuous symptoms of the diseases were the dot-like lesions on the under surface of the leaflets, which were visible 6 days after inoculations. These lesions had no definite shape, but varied in size and in the degrees of necrotic tissue present in their centers (Figure 7). On the lower surfaces of the leaves, a shiny green color was observed in areas near old lesions, instead of the normal opaque green color.

Leaves inoculated at older stages produced no visible symptoms of the disease and developed to normal size.

Almost immune clone FX 2831, Stage I leaves, when heavily inoculated produced black exudate both on the leaflets and the petrioles. These leaves soon lost color, blackened, shriveled and fell within a week. Lightly inoculated leaves produced very little black exudate, formed some raised lesions with light-green areas, and later some necrotic centers. The lesions were irregular in shape, both depressed and raised on the upper surface of the leaves. As the leaves increased in size, the diseased leaves became wrinkled and stunted. Leaves inoculated at Stages III and IV produced similar symptoms. Depressed lesions with light- to dark-brown necrotic areas and with or without yellowish-green margins were formed on the lower surface of the leaflets. These lesions had no definite shape, and some coalesced, with a light-yellow center of dead tissue surrounded by a black-brown necrotic border and a light-green halo. No stromatic or pseudostromatic tissue was observed on the surface of the lesions. Some very small pin-point lesions were seen near some secondary veinlets, or the

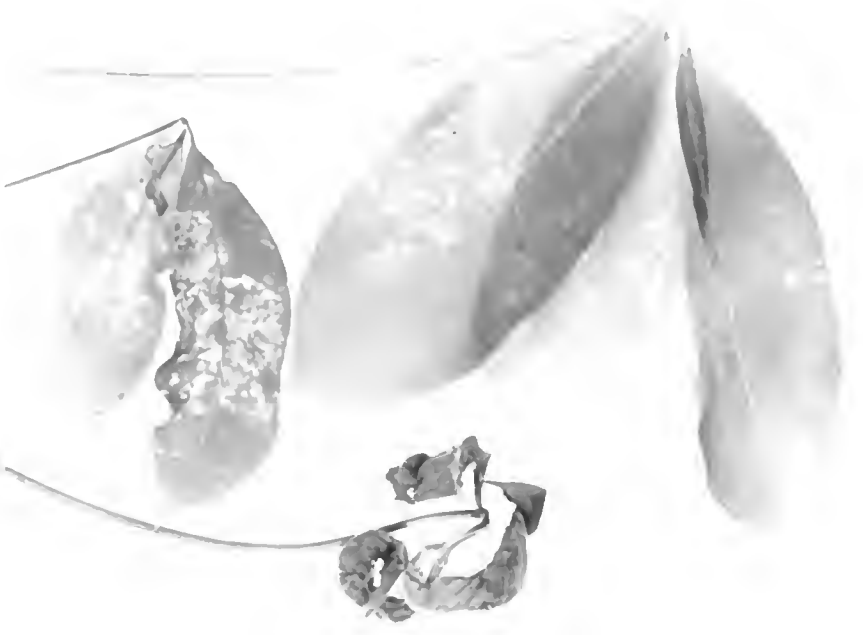

Figure 7. Stage III leaves 1 week after inoculation showing the extent of leaf malformation in highly susceptible clone Tjir 1 x Tjir 16. Resistant clone IAN 45-873. Almost immune clone P-122.

midribs of the leaflets. On the upper surface of the leaflets, small raised, necrotic lesions with no definite shapes were observed.

Almost immune clone P-122 leaves inoculated at Stage I produced black exudate similar to the one formed on other resistant and susceptible clones. As the leaves began to unfold, they showed slightly depressed areas on the lower surface of the leaflets, with dot-like necrotic centers. Some leaves with black exudate were misshapen, and were somewhat wrinkled and slightly smaller than healthy ones. The lesions did not continue to enlarge with further leaf development, and only the dead tissue in the centers of the lesions increased. No sporulation or stromatic structures were observed on the developed leaflets. The only visible symptoms observed were pin-point lesions surrounded by shiny green areas, instead of the normal opaque-green color of the under surface of the leaflets (Figure 8).

All of the lesions were slightly to deeply depressed with brownish-red areas. When magnified the brownish red-areas appeared as a group of individual cells, similar to small pebbles, surrounding an orange center. Lesions on the upper surface of the leaflets were slightly raised, giving the leaflet a wrinkled appearance. Under magnification the wrinkled surfaces of the leaves were not conspicuous, and the only visible symptoms were the translucent white lesions with pin-point necrotic centers. No lesions were observed on the leaf petrioles or midribs, and no sporulation or signs of the fungus were observed on the lesions produced on this clone.

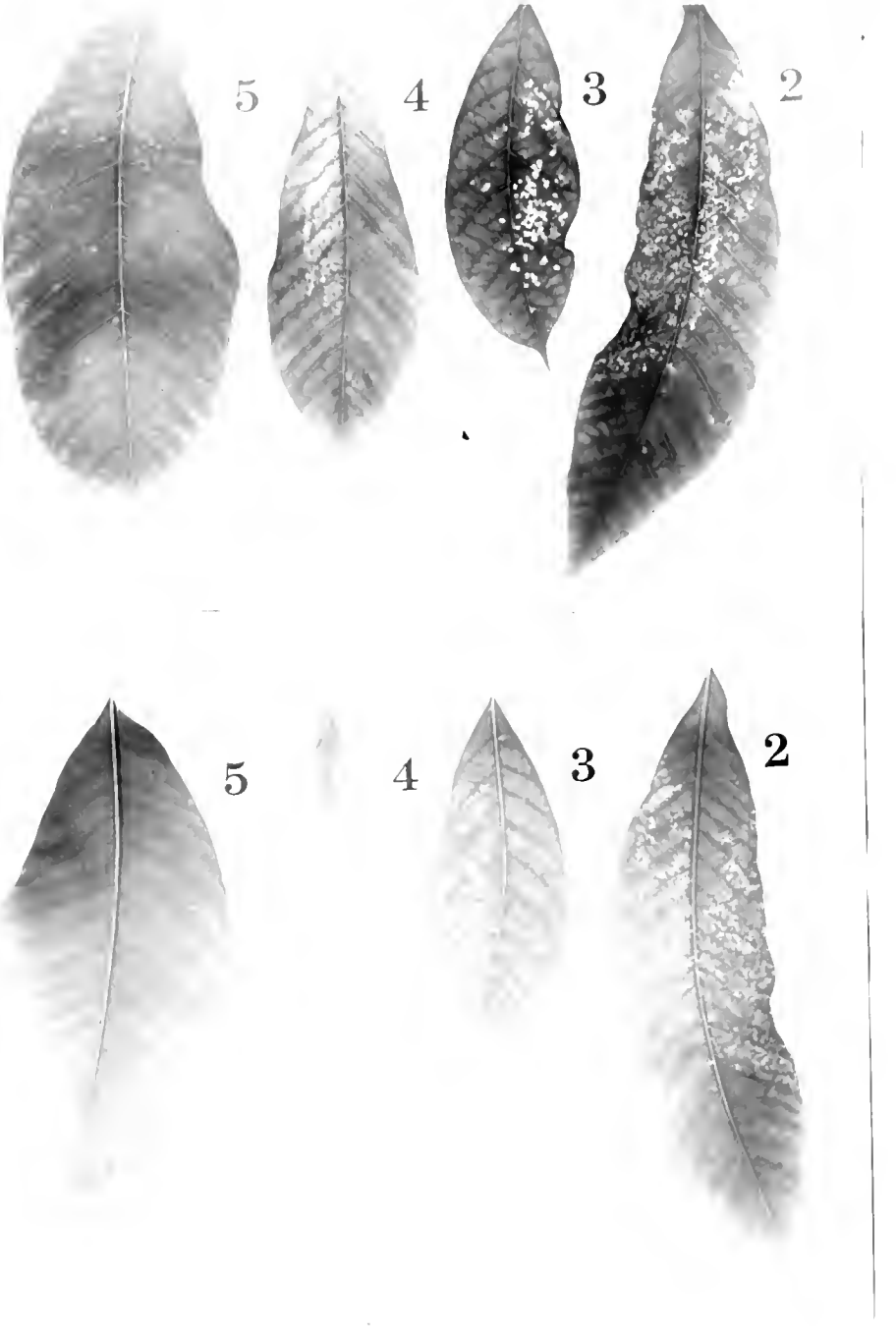

Figure 8. Top (A) and bottom (B) Stage III leaflets of (2) Resistant clone IAN 45-873, (3) Resistant clone FX 232, (4) Almost Immune clone FX 2831, (5) Almost Immune clone P-122.

HISTOLOGICAL STUDIES

The results of fungus infections in terms of the efficiency of fungus nutrition and the range of congeniality of host-parasite relationship of Dothidella ulei P. Henn. and the Hevea rubber are very similar to those of invasion by many of the so-called obligate parasites.

The establishment of a parasite will result after a successful penetration of a fungus germ tube when the conditions within the host are favorable. In many cases it has been observed that a fungus spore will germinate and invade the tissue even of a resistant host, but that the establishment of the parasite then fails and the fungus dies.

Martin (18) stated that the hyphae of yellow rust (Puccinia glumarum (Schm.) Eriks and Henn. will penetrate the stomata of resistant wheats but that further progress of the fungus is prevented by some unfavorable condition within the host. He further stated that on barley the mildew of wheat (Erysiphe graminis D.C.) will form haustoria, but after a few days the lack of receptivity of the host brings about degeneration of the fungus. The conditions which affect further growth of a fungus must be presumably of a chemical or physiological nature, and the ability of a fungus to thrive after penetration will be dependent on the presence of the required food material in the plant tissue.

Although many cytological studies have been conducted

frequently on the host-parasite relationships of various pathogenic fungi, especially in the order Uredinales (1,26,29), little attention has been given hitherto to similar studies within the order Dothideales group of which Dothidella ulei is typical.

In early studies of the South American leaf blight disease of rubber caused by D. ulei, Griffon and Maublanc (8) suggested that fungi of genera (Fusicladium, Aposphaeria, and Dothidella) previously found associated with the disease might actually be a single genus and not 3.

Stahel (25) investigated thoroughly the various phases of fungus infection, and by tracing mycelial strands was able to conclusively prove that the 2 other stages frequently found with the perithecia of Dothidella were a part of its life cycle.

Stahel's work laid the foundations for host-parasite relationship studies, especially with reference to details of cuticle penetration and the early phases of establishment of the parasite. However, he did not consider the distinctive phenomena of parasitism and pathogenesis in the resistant and immune trees.

The objectives of the present study was to compare the host-parasite relations between resistant and susceptible Hevea clones that might account for their disease reactions, and to determine any histological differences.

Method of Inoculation

Spores used for inoculation were obtained from diseased leaves of a highly susceptible clone Tjir 1 x Tjir 16 used in previous investigations (2). They were collected from sporulating leaf lesions with

a wet camel-hair brush and inoculation was made by brushing them on the moistened surface of young healthy leaves. It was necessary to cover the inoculated tree with a plastic bag unless the humidity in the greenhouse was high.

Leaves of the susceptible clone Tjir 1 x Tjir 16, resistant clones IAN 45-842, FX 232, and immune clones P-122, FX 2831, were inoculated at leaf Stages I, II, III, IV, V, and VI, and the ecological data recorded.

Inoculation experiments were carried out in 2 ways, in one a leaf was heavily inoculated with high concentrations of conidia (200 conidia per drop of water), and in the other a leaf was lightly inoculated with a less concentrated conidial suspension (40 conidia per drop of water).

Prior to inoculation each tree was thoroughly washed with a fine spray of tap water from a mist sprayer and gently shaken to remove the larger drops of water.

The conidia were brushed on the upper leaflet surface in one experiment, and on the lower leaf surface in a second experiment. After inoculation the entire tree was covered with a plastic bag for 2 or 3 days until the first macroscopic symptoms were visible. During the summer when the greenhouse vents were open the plastic bag was kept on until after conidia had been produced and collected.

Histology

Infected leaf material was collected every 3 hours the first day after inoculation and at 12-hour intervals thereafter and fixed in chemical solutions. The date and hour of fixation were recorded and

each collection, with the fixed material, was labeled accordingly.

In preliminary experiment, various fixatives were tried in combinations with different methods of dehydrating and staining. Best results were obtained with Newcomer's cytological fixative (20) with the following formula (by volume): 6 parts isopropyl alcohol, 3 parts propionic acid, 1 part ether (petroleum), 1 part acetone, and 1 part dioxane. The tertiary butyl alcohol of Johansen's (11), was employed for dehydration and embedding. Sections were cut with a rotary microtone 8 u thick. Heidenhain's iron alum hematoxylin was a satisfactory stain for early stages because it brought out the infection hyphae clearly. A safranin-fast green method was best for differentiating advanced stages of the fungus. Duplicate slides were stained only with safranin in advanced stages of disease development.

Various methods of leaf clearing were tried in combination with different staining methods. Best results were obtained with young tissues by clearing in a saturated solution of chloral hydrate preceded by fixation and killing in Newcomer's fixative. Staining was accomplished by gently warming the tissue on a glass slide flooded with a 1 per cent cotton blue in lactophenol for 2 minutes, counterstained with a 1 per cent acid fuchsin rinsed in 50 per cent alcohol, mounting in chloral hydrate and ringing with Clearcol mounting medium.[1]

This method was well adapted for studying certain stages of

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[1]Clearcol mounting medium. H. W. Clark, 33 So. High Street, Melrose 76, Mass.

conidial germination, direct cuticular penetration, and the establishment of the fungus beneath the cuticle, as it afforded a means of observing these phenomena in toto on relatively large areas of leaf surface.

Pieces of leaves were collected 6, 12, 18, 24, 36, 48, 60, 72, 96, and 144 hours, respectively, after inoculation, fixed in Newcomer's solution (20), strained, and mounted as previously described.

Early Stages of Infection

The germination of conidia, formation of appressoria and penetration of the cuticle, as observed in cleared leaves examined in toto, appeared to be similar in all the susceptible, resistant and almost immune clones. Following penetration, however, there were distinctive differences in the extent and nature of mycelium development in the susceptible, resistant, and almost immune clones.

At the end of 12 hours, the germination of most of the conidia had begun. The conidia were 2 celled, with the distal cell expanding and developing a slight protuberance. The conidial wall, however, remained intact, forming a thin slightly stained membrane about the protuberance.

After 24 hours, germination was well advanced, with rudimentary germ tubes (usually from the distal cell), penetrating directly through the cuticle and epidermal layer of cells. In a large number of cases appressoria were observed closely adhering to the cuticle.

Occasionally a germ tube branched and formed 2 distinct appressoria, each of which functioned in actual penetration of the

host. Penetration from the proximal cell of the conidium was generally observed after penetration from the distal cell had occurred. In some cases penetration was observed to take place without the formation of a germ tube or appressorium.

In many cleared leaves it was possible to observe direct penetration of a primary hypha regardless of location on the leaflet.

The primary hypha stained with cotton blue, revealing a cross-wall between it and the germination tube.

The single celled, irregularly shaped, primary hypha found in most 24 hour sections, rapidly branched out in all directions.

After 48 hours cells near the penetration hypha, stained with cotton blue, showed a granular condition of the protoplasm, and eventually collapsed. At the end of 48 hours the development of the fungus in leaflets of susceptible clones was more pronounced than that of resistant and almost immune clone leaflets. There was a considerable amount of granular protoplasm near the penetration sites, in leaflets of resistant clone FX 232. In almost immune leaflets of clones FX 2831 and P-122, the granular condition of protoplasm surrounding penetration sites was very pronounced, with a manifestation of necrosis that will be explained later. The position of these epidermal cells was very irregular, generally occurring in the highly susceptible clone leaflets 48 to 60 hours after inoculation. Epidermal cell collapse was frequently observed in resistant and almost immune clone leaflets. In highly susceptible clones, the mycelium developed throughout the leaflet regardless of type of host cell encountered. It was possible to observe the mycelium crossing over and

under, and surrounding conductive tissues.

Mycelial development was strictly intercellular, and in no instance was it observed to become intracellular. In leaflets of all clones tested the mycelium surrounded the subepidermal layer of cells, regardless of stage of leaf development.

In resistant and susceptible leaflets mycelial development was accompanied by the formation of a yellowish material, which is believed to be a reaction of the host tissue. The yellowish material was observed in advanced stages of infection of susceptible leaflets, next to collapsed cells, and near the point of infection. In resistant and almost immune leaflets the yellowish material was formed wherever mycelium was present.

In susceptible leaflets the mycelium radiated centripetally from the infection initial into the parenchyma layer after 96 hours, and formed a depressed area in the leaflet which was the first visible symptom of infection.

In resistant and almost immune leaflets, the average diameter of the lesions was much smaller, lacking any depressed area. However, the number of killed cells was greater than in susceptible leaflets, and the lesion could only be discerned as a pin-point.

Host Penetration.--The germinating conidia of Dothidella ulei penetrated the cuticle of the Hevea leaflets directly. This process was similar in all details for all clones. There was no cytological evidence to show that a cuticular barrier of any kind hindered the establishment of the fungus on any clones. Such penetration always occurred at a juncture epidermal cells, or at the base of a trichome.

No peeling back or mechanical rupturing of the epidermal layer and cuticle were observed (Figure 9).

Germination of Conidia.--Present observations of conidial germination on the host tissue were in accord with reports by Kuyper (14), Stahel (25), and Blazquez and Owen (2). A germ tube was first produced by the distal cell of the conidium, growing to about 200 u in length. The proximal cell would then produce a germination tube which developed similarly.

On susceptible leaflets the germination tube would grow to about 700 to 800 u in length, forming an arc and producing an appressorium when touching the surface of the leaf cuticle. Generally the germ tubes that were observed to penetrate susceptible leaflets directly were not as long as those forming appressoria.

In resistant leaflets from FX 232 and IAN 45-873 clones, as well as in leaflets of almost immune clones P-122 and FX 2831, the germination tubes were very short, not over 200 u, and sometimes penetrated directly through the cuticle. Most germ tubes formed appressoria upon coming in contact with the leaflet surface instead of penetrating directly.

Formation of an Appressorium.--As the advancing tip of the germ tube touched the surface of the leaflet, it became closely adhered, and developed into a more or less clearly differentiated appressorium. The appressoria in cross section appeared to be round or oval in outline and densely filled with cytoplasm, staining heavily with safranin, or hematoxylin. A larger number of appressoria were formed on leaflets of resistant and almost immune clones than on

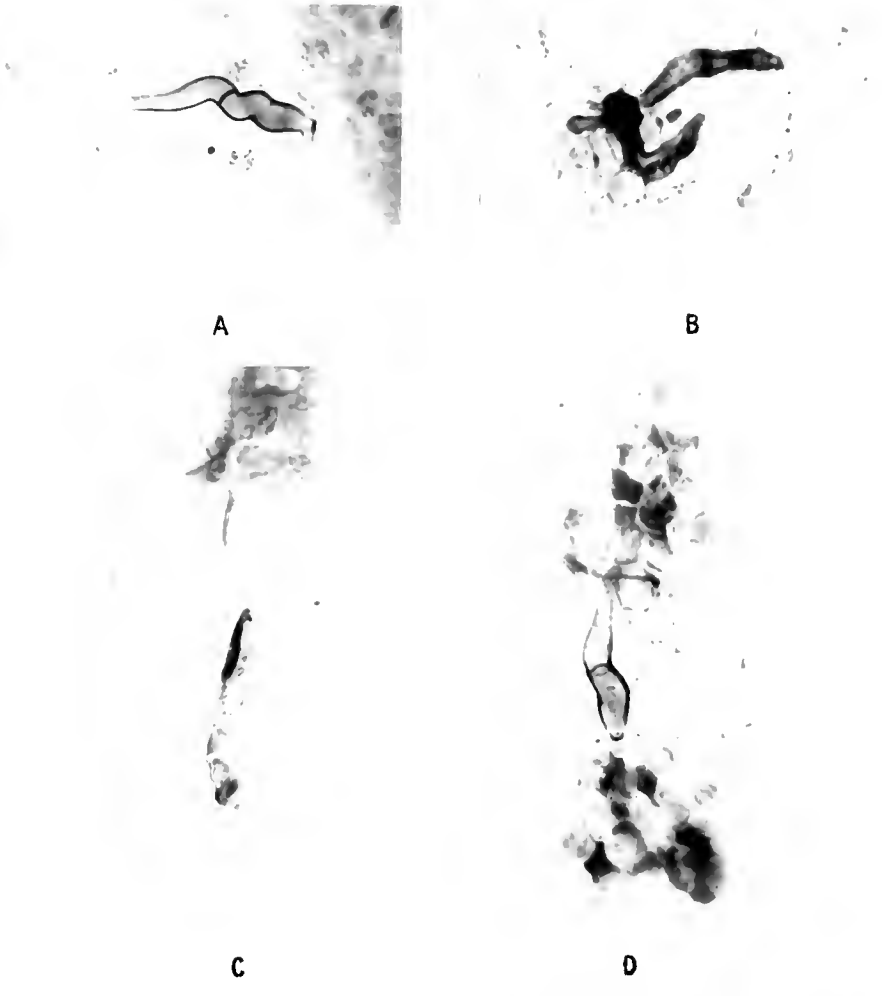


Figure 9. Photomicrographs of cleared leaves showing (A) Direct penetration of epidermis from young susceptible leaflet, (B) Penetration of epidermis from a FX 2831 resistant leaflet, (C) Direct penetration of epidermis from a FX 232 resistant leaflet, (D) Direct penetration of epidermis from a P-122 highly resistant leaflet.

leaflets of susceptible clones. In some instances conidia did not form a germ tube, but formed instead an appressorium immediately after germinating. The appressoria were apparently held fast to the leaflet surface by a mucilagenous sheath. The fixed remains of this structure were usually found in the cytological preparations.

Primary Hypha.--The term primary hypha is used to designate the first hypha that is visible within the epidermal layer of cells after penetration.

In host tissue, after penetration, the fungus developed an irregular, shapeless somewhat branched primary hypha. In the majority of cases the only difference between a germ tube and a primary hypha was a cross wall, and the affinity of the primary hypha for stains.

In susceptible leaflets the primary hypha was observed to be adjacent or surrounding 1 or 2 epidermal cells showing a granular protoplasm condition without a nucleus.

In resistant and almost immune leaflets all cells surrounding appressoria showed disorganized protoplasm, and no nuclei. Cells in the centers of older infections had a heavy yellow granular appearance, and in leaf Stages I, II, III, and IV, the cells were beginning to collapse (Figure 10). Primary hypha were not easily visible due to this rapid cell reaction.

Development of Infection

Two types of infections were observed on both the upper and lower epidermis, palisade and mesophyll layers of Hevea rubber leaflets. The first type was typical of susceptible leaflets, and the

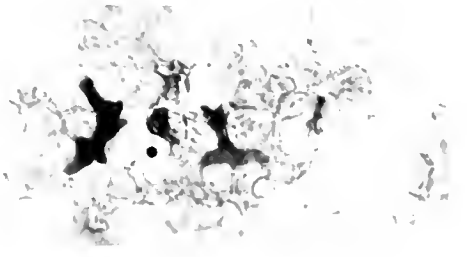


Figure 10. Collapsed cells in the center of a resistant leaflet lesion.

second type was typical of resistant and almost immune leaflets (Table 4).

Infection of Susceptible Leaflets.--Following penetration of the epidermis of susceptible leaflets and the formation of a primary hypha, 1 or 2 subepidermal cells changed in appearance. The primary hypha developed intercellularly, branched profusely, formed normal septate mycelium, which rapidly penetrated deep into host tissue. Vascular and sclerenchyma tissues did not hinder mycelial development as the mycelium was often observed to grow adjacent to vessels and sieve tubes (Figure 11).

Susceptible leaflets inoculated at Stages I and II were rapidly invaded by the mycelium after 96 hours, and after 120 hours mycelial hyphae had broken through the epidermis and cuticle forming conidiophores and conidia. Host cells near rapidly expanding hyphae were as normal as those beyond the area of infection. Leaflets inoculated at Stage I produced a black exudate near the point of infection, which adhered to the surface of the laminae and prevented normal leaflet development.

The condition of chloroplasts was used to determine the degree of deterioration of subepidermal cells. A more delicate indicator of health according to Rice (24) would be the amount and condition of chlorophyll present. Leaves which showed yellow, translucent areas around water-soaked infection loci at the time of infection, were sometimes found to have intact chloroplasts in all but the centers of the infection loci. Thus the chlorophyll disappeared before the chloroplasts lost definition. In all leaf stages chloroplast disintegration

TABLE 4

DEGREES OF RESISTANCE OF HEVEA CLONES TO *D. ULEI*
AND AMOUNT OF FUNGUS SPORULATION
According to Langford (15)

Clone	Sporulation	Degree of Resistance
Tjir 1 x Tjir 16	++++	10. Very highly susceptible
IAN 45-873	++++	8. Susceptible
FX 232	+++	5. Resistant
FX 2831	0	3. Highly resistant
P-122	0	2. Very highly resistant (Langford's immune)

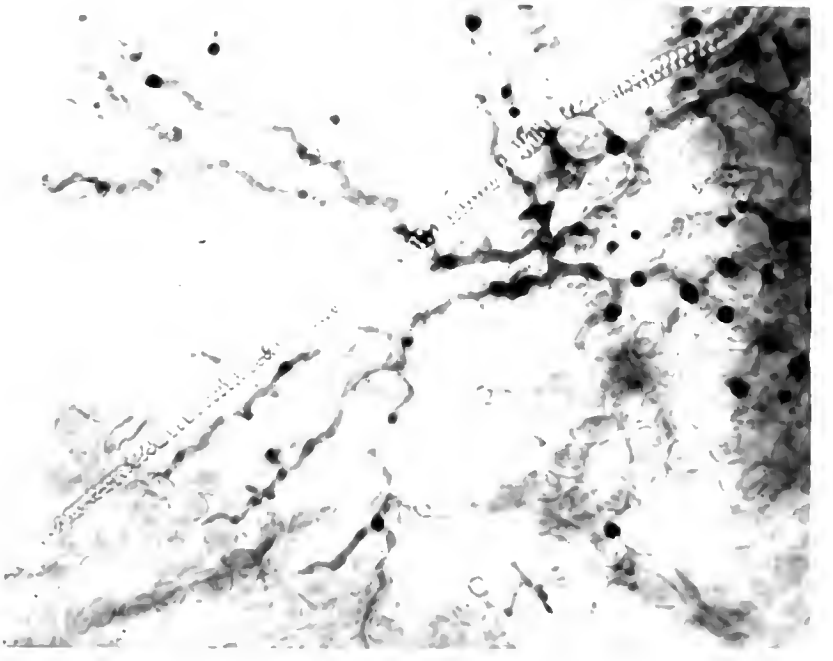


Figure 11. Cleared leaf section of a young susceptible leaflet soon after penetration, showing primary hyphae branching profusely and growing intercellularly.

preceded the formation of a yellow granular condition of the protoplasm. There were normal gradations in degeneration, from completely cleared, though well-oriented turgid cells in the centers of infections, to cells with chloroplasts coalesced but with the outlines of the individual plastids still visible, to cells with well defined, healthy chloroplasts.

Fastest mycelial growth in the earliest stages of infection appeared to be near the lower epidermal cells. This was observed in all the leaflets inoculated, regardless of whether they were from resistant or susceptible clones.

A yellow granular condition was observed near the point of infection, and throughout the parenchyma layer adjacent to the epidermis. Cell collapse began at the point of infection, and continued until both the mesophyll and the parenchyma layers were completely collapsed at the initial point of infection. This collapse produced abnormal tensions within the cell layers and formed an irregular depressed area. Conidial production was observed soon after conidiophore formation on the lower epidermis (Figure 12). After sporulation, the leaflet tissue appeared disorganized, wrinkled, dry, and finally dead.

Leaflets inoculated in Stages III, IV, and V showed that the tissues were readily invaded by the fungus in the early stages of infection; however, with continued leaf growth the areas of infection became somewhat delimited. The fungus was well developed throughout the mesophyll and pallsade layers of the leaf laminae, and conidial production began 108 hours after inoculation. In some instances sporulation was first observed in the outer perimeter, and later throughout

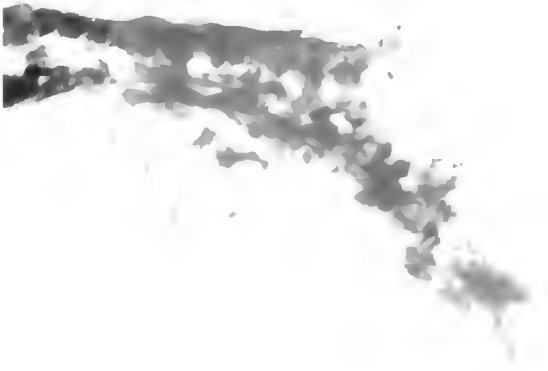


Figure 12. Cross section of a young susceptible leaflet section 108 hours after infection. Conidial production has begun, and both the palisade and parenchyma layers have collapsed.

the entire lesion on the lower surface of the leaflet. Some conidia were produced on the upper surface during later stages of the disease.

With the decrease in sporulation some stromatic initials were observed at the point of infection, increasing in size until they covered the surface of the lesion. Griffon and Maublanc (8) called these cells chlamydospores. When the lesion was larger than 1/4 of an inch the central cells were often killed, and in these instances the stromatic masses formed on the perimeter of the lesion. A few small round erumpent structures were observed on the upper epidermis directly above the stromatic mass. These structures were considered to be spermogonia by Stahel (25) and Langford (15). These spermogonia were filled with spermatia, which oozed out through a distinct pore (Figure 13) approximately 2 months after inoculation.

In a tangential section of a leaflet 2 months after inoculation, stained with safranin it was possible to observe a deeply stained tannin-like material. In a cross section the intra- and intercellular presence of the yellow material was observed in the sylem vessels (Figure 14).

In lesions 1 year old, the mycelium was dark brown, with definite septations, and grew intercellularly (Figure 15). A large part of the stromatic masses was growing on the surface of collapsed or semi-collapsed tissue. Spermogonia appeared to be empty, and no spermatia were visible in any of the sections prepared.

The perithecial stage was not observed.

Leaflets inoculated at Stage VI showed similar fungus penetration to earlier stages. However, fungal development was not as

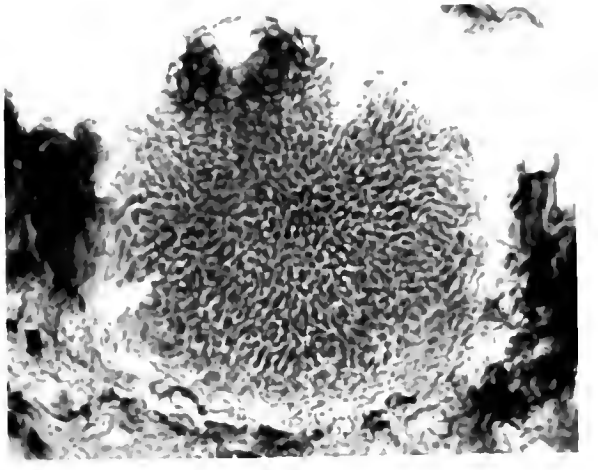


Figure 13. Cross section through a spermogonium, showing the spermatia and the distinct pore (ostiole).

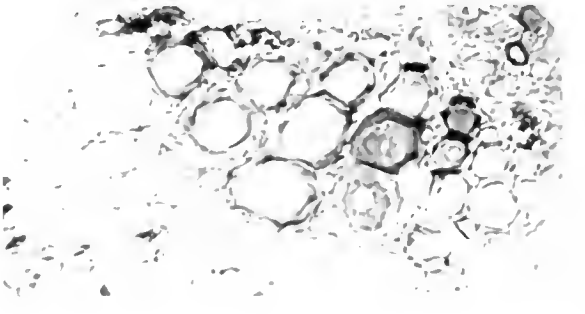


Figure 14. Cross section of an old diseased susceptible leaflet showing yellow material plugging the xylem vessels.

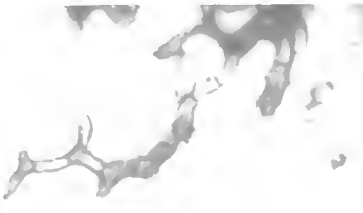


Figure 15. Cleared leaf section of a 1-year-old diseased leaflet showing dark-brown mycelium with typical septations.

vigorous, there was less severe impoverishment of underlying host tissues, and a decreased amount of necrosis. Cross sections showed mycelial invasion of the mesophyll and palisade layers 2 weeks after inoculation. The striking difference observed was the lack of conidial production and the formation of stromatic tissue. Impoverishment of the palisade layer appeared in the middle of the lesion 12 to 15 days after inoculation manifested chiefly by the disappearance of plastids and by marked vacuolation.

Infection of Resistant Leaflets.--Host reaction to the fungus invasion of IAN 45-873 and FX 232 leaflets was very similar, and they therefore were considered as one type of reaction and will be described together.

The immediate host reaction to fungus penetration was the distinctive difference between susceptible and resistant foliage. Epidermal and parenchyma cells of susceptible leaflets appeared to be normal near primary hyphal development, while in resistant leaflets 1 or 2 epidermal cells collapsed, and a few parenchyma cells adjacent to primary hypha appeared disorganized and soon collapsed (Figure 16).

In resistant clones, 108 hours after inoculation the fungus had developed throughout the various leaflet tissues. Cells adjacent to mycelium showed some impoverishment with cell disorganization more pronounced in the area nearest the point of infection.

A very pronounced host reaction of the resistant and immune clones was the production of a yellow material found throughout areas where fungus hyphae had invaded the tissues. In most of the disorganized cells there was a granular appearance, yellowish in color,

Figure 16. Collapsed epidermal cells near the point of infection of resistant FX 232 leaf tissue.

which changed to dark brown in older lesions.

The subepidermal parenchyma of leaflets of clone IAN 45-873 showed protoplasm disorganization 108 hours after infection. Mycelial hypha were well distributed throughout the subepidermal parenchyma cell layer 6 days after infection.

The fungus continued to spread throughout the tissue, and after 7 days most of the parenchyma layer of the mesophyll and some palisade cells nearest the point of infection had been invaded. Parenchyma cells closest to infection points showed disorganized chloroplasts and the appearance of yellow granular material. The entire mesophyll layer was invaded 10 days after inoculation, and after 12 days the palisade layer showed an impoverished granular condition.

Stromatic tissue appeared on the lower surface of the leaflets near the apparently dead cells, and after 13 days, condiophores and conidia were formed in abundance. They were also formed on the upper surface of the leaflet, directly over the discolored epidermal and palisade cells. Spermogonia were not observed in any of the inoculated leaflets.

Diseased tissues of FX 232 and IAN 45-873 were similar except for a decrease in sporulation, intercellular invasion, and necrotic tissue in clone FX 232.

Almost Immune Clones.--Histological observations of leaflets considered to be almost immune revealed that the fungus was capable of penetrating, colonizing, and killing leaflet tissue. The type of host reaction observed was similar to the resistant type reaction.

Observations of almost immune (hereafter designated as highly resistant) leaflets will be described, and essentially considered to be of the resistant type of host reaction.

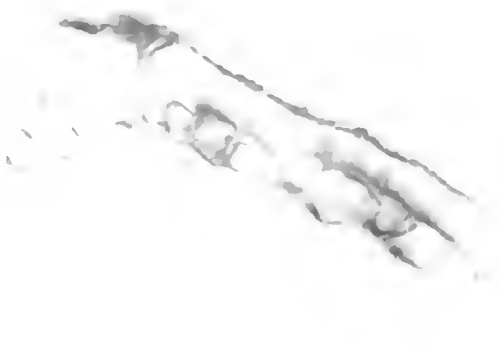
Host reactions and tissue invasion of clones FX 2831 and P-122 were considered similar and are described together. In these 2 clones inoculations of leaflets at Stage I produced a black exudate on or near the lesions within 36 hours (Figure 17 A). Epidermal cells near cell junctures where infection hyphae penetrated collapsed soon after penetration occurred (Figure 16). Rapid proliferation of the fungus mycelium throughout the subepidermal layer was observed on the lower surface of a leaflet inoculated 3 days previously. The host nuclei in the underlying mesophyll cells retained their normal shape and were not as heavily stained as the cells of the subepidermal layer (Figure 17 B).

In cleared leaf sections a yellow material was observed intercellularly throughout the diseased tissue. Collapsed cells appeared yellow to brown before staining, and stained a dark blue with cotton blue, and deep red with safranin (Figure 18 C)

Leaflets inoculated on the upper surface showed collapsed palisade cells after 3 days, mesophyll invasion occurred after 9 days, and a total collapse after 2 weeks.

Highly resistant leaflets inoculated at Stage II had similar reactions as Stage I leaflets, except that cell collapse was not as rapid or as extensive. Fungus growth appeared to be restricted to the subepidermal layer for a longer period of time.

The striking differences between highly resistant and



A

B

Figure 17. Cross section of a P-122 highly resistant leaflet showing (A) A laticiferous cell, (B) A black exudate near an infection point and the complete surrounding of the subepidermal layer of cells.

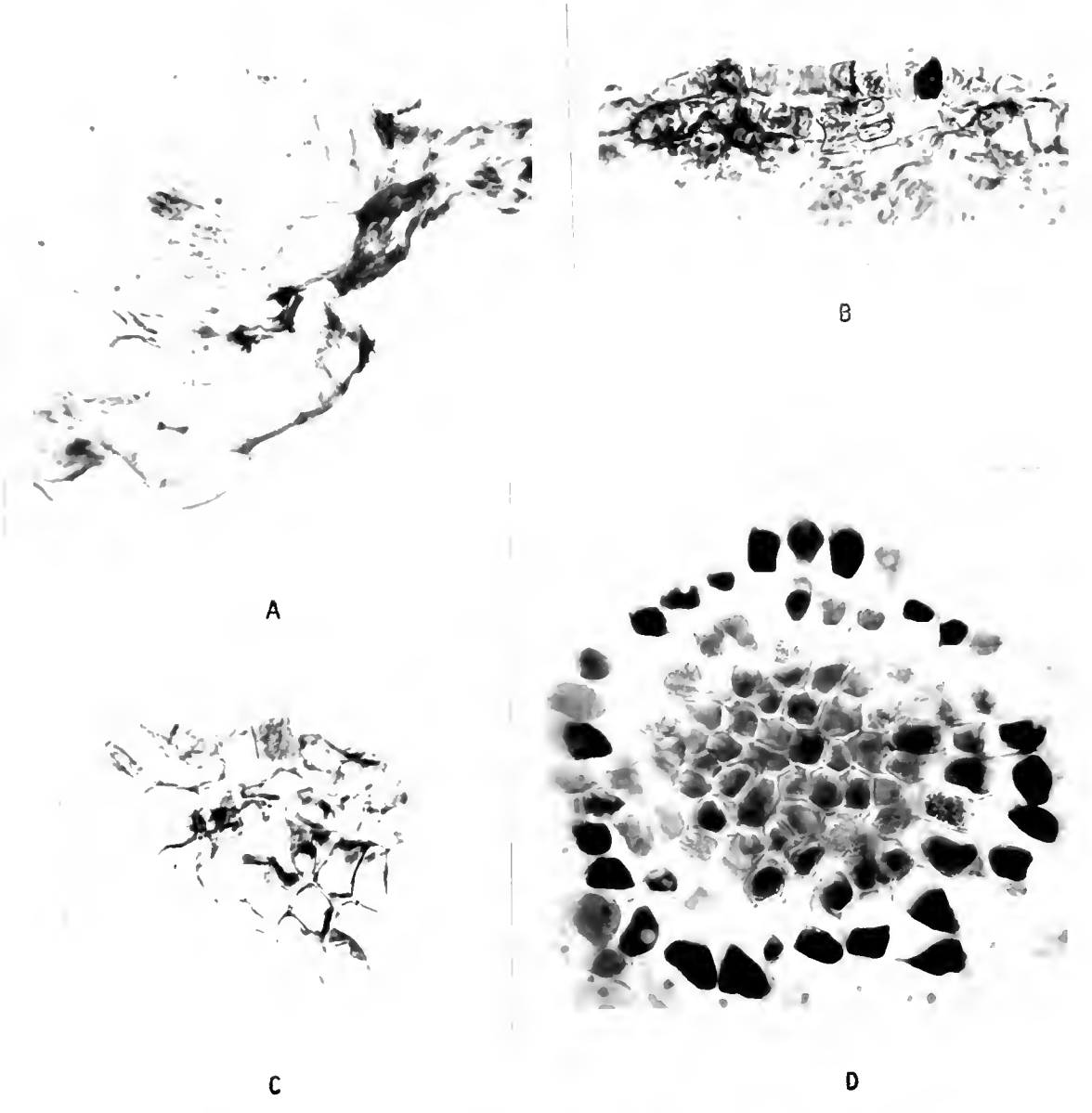


Figure 18. Photomicrographs of resistant host reaction to invasion of D. ulei. (A) Collapsed mesophyll and palisade layers showing necrotic and disorganized cells. (B) Cross section of a lesion from a highly resistant leaflet showing sclerenchyma-like cells. (C) Yellow material observed near mycelium of invaded resistant cells. (D) Cells with a granular yellow appearance in the center of the lesion, and dark sclerenchyma-like cells found in the perimeter of the lesion.

susceptible clones were the lack of conidiophores and conidia production, the absence of leaf distortion, and the failure of the fungus to produce stromatic tissues in the highly resistant clones (Figure 18 A).

Six months after inoculation, mesophyll tissue appeared to be normal, and only cells near the point of infection had a granular yellow appearance. Cells near the center of the lesion were dark, tightly packed and associated with sclerenchyma tissue (Figure 18 D).

Leaves inoculated at Stages III, IV, and V also show these types of cells, which appeared in a circular pattern surrounding a point of infection, or scattered throughout the leaflet (Figure 18 B). Cells adjacent to the sclerenchyma-like tissue and in the subepidermal layers were impoverished. The yellow material observed in younger lesions was not observed in older lesions.

Slight depressions were observed on the outer perimeter of the lesion, but generally there was no alterations of cellular structures observed.

On the upper surface of the leaflets, epidermal and palisade layers were normal in shape and yellowish-brown. Cells adjacent to the palisade layer contained impoverished chloroplasts. In some cases large lesions were observed to have parenchyma-like palisade cells, instead of normal palisade cells.

No stromatic structures or fungus fructifications were observed in the lesions of almost immune leaflets. No specific mechanical tissues were observed to be formed in resistant and highly resistant leaflets.

In highly resistant leaflets inoculated in Stages III to VI no large development of a lesion was observed. Generally epidermis penetration occurred, but no further fungus development was observed. In Stage III leaves, a few cells appeared to be impoverished in a small area surrounding the point of infection but did not stain with safranin.

BIOCHEMICAL STUDIES

In early microchemical work Rawlins (22) stated that microchemical methods could be applied to identify resulting compounds from fungi grown in synthetic culture media, as well as in the cells of the pathogen or host.

With the rapid advances being made in the field of chromatography and by use of newer ion exchange resins for the purpose of concentrating the samples, small volumes of plant extracts may be used to find significant differences between healthy, diseased, resistant, and susceptible plants.

Kuc et al. (14) studied the production of fungistatic agents by potatoes in response to inoculation with Helminthosporium carbonum. They prepared ethyl alcohol extracts of healthy and diseased potato peel and pulp tissues. Part of the extracts were used for chromatographical analysis and part were added to potato-dextrose agar media.

Fife (6) found through the use of paper chromatography striking differences in the relative concentrations of certain amino acids in the juices expressed from healthy and diseased beet leaves, and in the phloem exudates collected from healthy and diseased sugar beet roots.

Zscheile and Murray (32), using paper chromatography, found specific differences of the amino acids and amounts present in wheat ovules in relation to genes for disease resistance.

Blazquez and Owen (2) grew the fungus D. ulei on media prepared

from water extracts of Hevea brasiliensis Muell. Arg. leaves. They reported that both l-inositol and quebrachitol (mono-methyl ether of l-inositol) greatly favored fungous growth in small concentrations (100 mg. per liter), but that at high concentrations (above 400 mg. per liter) no fungous growth was obtained. They stated that there may be a relationship between the toxic effects of high concentration of both compounds and disease development. Quebrachitol is present in the latex of species of Hevea in concentration of 0.5 to 2.0 per cent, and this high concentration of quebrachitol might account for the susceptibility of young Hevea leaves to D. ulei, whereas older leaves are immune or only slightly susceptible.

Resing (23) found that quebrachitol was only found in the water-soluble impurity fraction of latex lipids. Bolle-Jones (3) in the determination of sugars present in the laminae of Hevea brasiliensis found glucose, fructose, sucrose and relative large amounts of i and l-inositol, but not quebrachitol.

Media Containing Quebrachitol

In previous nutritional experiments where media were prepared with quebrachitol (2-mono-methyl ether of l-inositol) as a vitamin source, fungal growth was obtained in concentrations of 25 to 50 mg of quebrachitol per liter. Using chromatographic methods it was determined that the quebrachitol used in early experiments was not pure and that it contained small amounts of 3- and l- forms of inositol. It was therefore necessary to determine which of these 3 compounds, pure quebrachitol, i-inositol, or d-inositol, were utilized by the

fungus. An experiment was set up in which contaminated quebrachitol (with i- and d-inositol),[1] pure quebrachitol,[1] d-inositol,[2] quebrachitol,[2] and i-inositol were used as the only vitamin source in a semi-synthetic medium and galactose was used as the carbohydrate source. The semi-synthetic media was prepared as in previous experiments (2), with the vitamin source being used at a concentration of 20 mg per liter, and the carbohydrate source at a concentration of 10 g per liter. The media was tubed, plugged and autoclaved.

On contaminated quebrachitol media black stromatic tissue developed on the surface of the slant with some olivaceous mycelium growing along the periphery of the colony. Conidial formation was moderate with fair spermogonial development. The average diameter of the colonies was 7 mm, and the average height was 5 mm after 6 months (Figure 19).

On pure quebrachitol medium the fungus produced white mycelium from the black stromatic tissue on the surface of the medium. Black stromatic tissue, either superficial or partly submerged grew on the medium. Conidial and spermogonial production was moderate. Colonies grew to 5 mm in diameter, and 6 mm in height within 6 months.

On the quebrachitol medium dark-brown to black stromatic mycelium grew on the surface of the medium, with whitish mycelium growing

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[1]Samples of contaminated and pure quebrachitol were obtained from Dr. O. D. Cole of the Firestone Tire and Rubber Company, Akron, Ohio.

[2]Samples of d-inositol and quebrachitol were obtained from Dr. H. J. Teas of the University of Florida, Agricultural Experiment Station, in Gainesville, Florida.

Figure 19. Six month old cultures of D. ulei comparing growth in media from (left to right) contaminated quebrachitol; pure quebrachitol (Firestone Co.); quebrachitol (Calif.); d-inositol; with l-inositol as control.

on the surface of the stromatic mass of hyphae. Conidial and spermogonial formation was very abundant. Colonies grew to 3 mm in diameter, and 3 mm in height after 6 months.

Dark-brown to black stromatic mycelium grew on the surface of the d-inositol media, with white mycelium growing from the periphery of colonies. Conidial and spermogonial formation was fair.

On the i-inositol medium mycelium developed on the surface and grew deep into the medium. Conidial and spermogonial formation was fair. The fungus colonies were compact and small. They grew to 3 mm in diameter and 2 mm in height within 6 months.

Media Containing Latex Serum

Resing (23) in experiments with phosphatides from fresh unammoniated latex found that quebrachitol was not present in the alcohol-soluble fraction and was only able to detect it in the water-soluble fraction of the latex. Smith (27) reported that quebrachitol was present only in the water-soluble part of latex.

An experiment was devised to determine the possible presence of growth-increasing substances (such as quebrachitol) in the water-soluble fraction of the latex (serum). Serum from latex (Firestone S-4) was obtained by a process of super-filtration as follows: A 3-foot length of 1 1/8 in. wide cellophane dializing tube was soaked in water for 10 minutes. One end of the casing was tied, the tube filled with latex and the 2 ends tied together. The loop was then hung over a glass rod and placed inside a bouyoucous cylinder. The casing was kept moist by placing a large beaker over the cylinder to maintain

high humidity. As the rate of filtration decreased, the casing was kneaded gently to remove a pasty deposit from its walls.

The 226.5 ml of serum obtained by super-filtration from 1.35 liters of latex was transferred to a flat glass dish inside a larger beaker containing 1N sulfuric acid. Both beakers were covered with a glass jar to remove any remaining ammonia from the serum portion of the latex. The serum fraction was removed from the flat dish with the sulfuric acid when ammonia fumes could no longer be detected. It was then divided into 2 fractions, a 100 ml fraction and a 140 ml fraction. The 100 ml fraction was used to prepare media with 5 different concentrations of serum and was sterilized by autoclaving at 15 lbs per square inch of pressure for 30 min. The 140 ml fraction was used to prepare media using 6 concentrations of serum and was sterilized by filtering through a sterile Seitz filter. In the first serum fraction, the serum was mixed with water agar in proportions which reduced the volume of serum from 50 ml to 1 ml per 100 ml of medium at intervals of 25, 10, 5 and 1 ml. No additional nutrients were added to the serum media. In the second fraction, the water agar in the desired concentrations, and the necessary glassware to mix and measure the media were washed with tap water, rinsed twice with distilled water and autoclaved. The mixing of sterile solutions was done in a transfer chamber under aseptic conditions, and the Erlenmeyer and graduated cylinders used were flamed before and after pouring the serum. Solutions were made up to volume, mixed, tubed, plugged and slanted.

Conidia seeded on media containing concentrations of 1, 5, and 10 ml of serum germinated readily, but on the 25, 40 and 50 ml

concentrations no germination was observed. In the 1 ml concentration mycelium grew from the surface of the stromatic tissue on the test medium. Olivaceous mycelium developed on the surface of the media and on the surface of the stromatic tissue. Conidial production was fair and spermogonial production was moderate. Colonies grew to 1 mm in diameter after 50 days, and to 5 mm after 300 days.

In the media prepared from autoclaved serum no fungus growth was obtained in concentrations above 10 ml of serum per liter.

Media with 10 ml of serum per liter produced light-brown to yellow pseudo-stromatic tissue in 50 days. Black stromatic tissue grew on the surface of the medium with olive mycelium developing on the stromatic tissue. Conidial production was fair. Spermogonial production was moderate. Colonies grew to 5 mm after 300 days.

Media prepared with 1 and 5 ml of serum gave similar fungus growth up to the 100 ml per liter concentration. Conidia germinated more readily and growth appeared to be more vigorous. Conidial production was fair and spermogonial production was moderate. Colonies grew to 5 mm after 300 days.

Effects of Carbohydrates on the Growth of the Fungus

Carbohydrates are of extreme importance in the nutrition of fungi as they are the main sources of carbon. Almost half of the dry weight of fungous cells consists of carbon. Protoplasm, enzymes, the cell wall, and reserve nutrients stored within the cells are compounds of carbon. In addition to being the main structural elements, carbon compounds play an equally important functional role.

Various fungi possess different abilities to use carbohydrates according to Wolf and Wolf (31). Lilly and Barnett (17) stated that the most common source of carbon reported was glucose. Most species generally gave better yields on hexoses than on pentoses, although Hawkins (9) found that Glomerella cingulata utilized 2 pentoses, arabinose and xylose.

Moore (19) reported that certain pathogens possessed wide capabilities for utilizing carbohydrates, whether mono-, di-, or polysaccharides. She determined that Phymatotrichum ommivorum used glucose, fructose, galactose, maltose, sucrose, lactose, and mannitol.

An experiment was designed to test the effect that various sugars might have on the growth of D. ulei. The sugars were tested in a basal semi-synthetic media which was prepared as follows: Carbon source, 10 g per liter; asparagine, 2 g; KH_2PO_4, 1 g; Mg^{++} ($MgSO_4 \cdot 7H_2O$), 0.5 g; Fe^{+++} ($Fe_2SO_4 \cdot XH_2O$), 0.2 mg; Zn^{++} ($ZnSO_4 \cdot 7H_2O$), 0.2 mg; M_n^{++} ($M_nSO_4 \cdot H_2O$), 0.1 mg; biotin, 5 ug; thiamine, 100 ug; 20 g of agar; and distilled water to make 1 liter. One-half liter of distilled water was first warmed and the various ingredients added until dissolved and made up to 1 liter. The carbohydrates tested and the concentrations per liter of the stock solutions were as follows: d-Mannitol, 75 and 150 g; d (+) Mannose, 75 and 150 g; d (+) Maltose, 75 and 150 g; d (+) Galactose, 75 and 150 g; l-Sorbose, 75 and 150 g; d (+) Xylose, 75 and 150 g; d (+) Arabinose, 75 and 150 g; d (+) Lactose, 75 and 150 g; d (+) Raffinose (Hydrate), 75 and 150 g.

Blazquez and Owen (2) studied the effects of vitamin-mino acid combinations on growth of D. ulei. They determined that l-inositol

- glutamic acid; nicotinic acid - glycine; riboflavin - glycine; and p-amino benzoic acid were the best combinations for fungous growth. With this in mind the following combinations of vitamins and amino acid, and concentrations per liters in 100 ml of stock solutions were prepared: i-inositol, 1.2 g; p-amino-benzoic acid, 15 mg; nicotinic acid, 600 mg; riboflavin, 15 mg; l-glutamic acid, 441.4 mg (added to the i-inositol stock solution); glycine, 222.5 mg (one lot added to the nicotinic acid stock solution and another added to the riboflavin stock solution). Vitamin-amino stock solutions were made up to 2.7 liter lots and 60 g of agar were added to each lot.

It was necessary to have 2 aliquots from each 2.7 liter lot since 2 concentrations of sugars were to be tested. Therefore each lot was separated into 2 aliquots of 1350 ml. From each aliquot 135 ml were placed into ten 250 ml Erlenmeyer flasks. Fifteen ml from each 75 and 150 g concentrations of sugar stock solution was added to the 135 ml Erlenmeyer flasks of the vitamin-amino acid aliquots. Thus the total volume in each flask was 150 ml, and the final concentrations of sugars were 5 and 10 g per liter. The final concentrations per liter of the vitamin-amino acid combinations were: i-inositol 400 mg; p-amino-benzoic acid, 5 mg; nicotinic acid, 200 mg; riboflavin, 5 mg; l-glutamic acid and glycine, 21 mg of nitrogen equivalents. One small lot of 150 ml of each vitamin-amino acid combination without sugars was used as controls. The prepared media were then tubed, plugged, sterilized, slanted and stored at 4° C. When conidia became available, 10 tubes of each sugar-vitamin-amino acid combination were planted and seeded with conidia. Germination readings were taken periodically and

compared with those of other media previously prepared. After conidial germination a small section of medium with germinating conidia was then transferred to slants of the same sugar medium. These slants were then incubated at 20° - 22° C. In order to evaluate the growth of conidial and spermogonial development after 80 and 280 days, an arbitrary numerical rating was used, with 4 being maximum and 0 minimum development.

Response of the fungus to the various sources of carbon tested in basal semi-synthetic media varied greatly. There was no significant difference between the vitamin-amino acid combinations riboflavin-glycine; nicotinic acid-glycine; and p-amino benzoic acid. Only the combination i-inositol-glutamic acid gave excellent growth with all the carbon sources tested (Table 5).

The pentoses, arabinose, xylose, and sorbose gave fair growth, while the hexoses, maltose, galactose, mannose, and the sugar alcohol, mannitol gave the best growth (Figure 20). The discharide, lactose and the trisaccharide, raffinose gave intermediate results between the pentoses and hexoses (Figure 21).

Leaf Extract from Hevea Spp.

Leaf extracts for susceptible, resistant, and highly resistand Hevea brasiliensis, and from H. benthamiana were prepared with distilled water and also with 80 per cent ethyl alcohol in a soxhlet apparatus. The extracts were prepared as follows: 2 g of leaf laminae were cut in small squares, placed in a No. 1 Whatman extraction thimble and the thimble inserted into the soxhlet extraction tube. One hundred ml of water were poured into an extraction flask, glass

TABLE 5

EFFECT OF VITAMIN AND AMINO ACID COMBINATIONS WITH SUGARS ON THE GROWTH OF D. ULEI

Vitamin-amino Acid Plus Sugar Medium	Spermogonial Formation		Conidial Production[a]		Av. Diameter of Colony (mm)	
	A[b] 5	B[c] 10	A[b] 5	B[c] 10	A[b] 5	B[c] 10
i-inositol-l glutamic acid plus						
Mannitol	1	1	2	3	7	5
Mannose	3	2	3	0	12	13[b]
Maltose	1	0	2	1	10[d]	10
Galactose	3	4	2	1	10	10
Sorbose	1	-	2	-	6	-
Xylose	3	0	2	3	6	5
Arabinose	0	0	1	1	6	3
Lactose	1	2	2	2	8[e]	10
Raffinose	0	0	2	0	7	5
Control	2	2	3	3	8	8
P-amino benzoic acid plus						
Mannitol	1	1	0	1	1	1
Mannose	1	2	0	0	6	3
Maltose	3	3	3	0	5	5
Galactose	0	1	0	0	2	3
Sorbose	-	-	-	-	-	-
Xylose	-	0	-	0	-	0.1
Arabinose	0	0	0	0	0.1	0.1
Lactose	3	2	0	3	3	3
Raffinose	3	3	0	3	5	3
Control	2	2	3	3	5	5
Nicotinic acid-glycine plus						
Mannitol	0	1	0	1	1	2
Mannose	2	2	0	1	3	3
Maltose	3	3	3	3	6	6.5
Galactose	1	1	1	1	5	4
Sorbose	-	-	-	-	-	-
Xylose	-	1	-	0	-	0.1
Arabinose	1	-	1	-	1	-
Lactose	3	2	3	2	2	2
Raffinose	-	3	-	0	-	0.5
Control	1	1	3	3	4	4

TABLE 5--Continued

Vitamin-amino Acid Plus Sugar Medium	Spermogonial Formation		Conidial Production[a]		Av. Diameter of Colony (mm)	
	A[b] 5	B[c] 10	A[b] 5	B[c] 10	A[b] 5	B[c] 10
Riboflavin plus						
Mannitol	-	2	-	2	-	2
Mannose	1	2	0	1	5	3
Maltose	2	1	3	3	5	5
Galactose	0	2	0	2	2	3
Sorbose	0	-	0	-	2	-
Xylose	1	-	0	-	1	-
Arabinose	2	0	2	1	2	2
Lactose	1	0	3	3	4	4
Raffinose	1	2	0	1	2	2
Control	1	1	1	1	3	3

[a]Arbitrary numerical rating for percentage of spermogonial and conidial formation: 4, above 75 per cent; 3, 50-75 per cent; 2, 25-50 per cent; 1, below 25 per cent; 0, none.

[b]Media prepared with 5 g of sugar.

[c]Media prepared with 10 g of sugar.

Figure 20. Nine-month-old cultures of D. ulei comparing growth in media prepared from (left to right) riboflavin plus sugars lactose 10 g; galactose 10 g; mannose 5 g; maltose 10 g; i-inositol plus lactose 10 g; galactose 10 g; maltose 5 g; and mannose 10 g.

Figure 21. Nine-month-old cultures of D. ulei comparing growth in media prepared from (left to right) nicotinic acid plus the sugars lactose 10 g; galactose 10 g; maltose 5 g; mannose 5 g; i-inositol plus lactose 10 g; galactose 10 g; maltose 5 g; and mannose 10 g.

beads were added, and the apparatus was assembled. The extraction process was of 1 hour duration. Alcohol extracts were made in the same manner. To the water extracts were added 5 g of agar and 0.5 g of malt extract, the extract solution was then made up to 250 ml. In the case of the alcohol extracts, it was necessary to reduce the volume to about 30 ml, water was then added to make 100 ml, the agar and the malt extract added and the solution made up to 250 ml. The leaf-extract media were tubed, plugged, autoclaved at 15 lbs of pressure for 30 min. and then stored until conidia became available for inoculation.

Susceptible leaf extract media.--Extracts were made and tested from a Far Eastern clone, Tjir 1 x Tjir 16, using 6 month old dry leaves; 36-day-old diseased leaves which had been inoculated when they were 6 days old.

Water extract from 36-day-old diseased leaves.--Black stromatic mycelium grew on the surface and deep in the medium. Olive and white mycelium were formed on the surface of the stromatic mycelium. Conidial and spermogonial production was good. Colonies grew to a diameter of 8 mm in 6 months (Figure 22).

Alcohol extract from 36-day-old diseased leaves.--Black stromatic mycelium was produced on the surface of the medium, with pseudostromatic tissue growing deep in the medium. Long thin hypha were produced from the black stromatic mycelium, with small patches of cottony-white mycelium in some areas. Abundant conidial and spermogonial productions were observed. Spermatia oozed out of the spermogonia in small droplets. Within 6 months colonies grew to 10 mm in

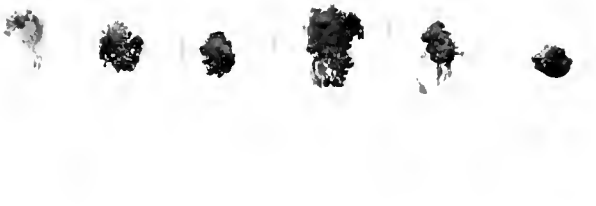

Figure 22. Six-month-old cultures of *D. ulei* comparing growth in media prepared from Hevea leaf extracts (left to right) ethyl alcohol from young, diseased susceptible leaves; water extract from young, diseased susceptible leaves; water extract from FX 232 leaves; water extract from young P-122 leaves; water extract from 6-month-old leaves; water extract from 2-month-old F 4542 leaves of an *H. benthamiana* clone.

diameter, and 4 mm in height (Figure 22).

Water extract from dry leaves.--Light-brown to yellowish mycelium was produced on the surface as well as deep in the medium. In some colonies dark-brown pseudostromatic tissue grew on the surface of the medium. No conidial or spermogonial production was observed. Colonies grew to a diameter of 0.01 mm in 80 days.

Dry leaves alcohol extract.--Light-brown to yellowish pseudostromatic mycelium was formed. Many colonies did not grow. No conidial and spermogonial production was observed. The average diameter of the colonies was 70 u after 80 days.

Resistant leaf extract media.--Leaf extracts from a resistant clone FX 232 were made with distilled water and 80 per cent ethyl alcohol.

On water extract, olive and black stromatic mycelium grew on the surface, with dark pseudostromatic tissue forming deep in the medium. Conidial and spermogonial production was good. The average diameter of the colonies was 7 mm after 80 days (Figure 22).

Highly resistant extract media.--Water and 80 per cent ethyl alcohol extracts were prepared from 4-day-old leaves, healthy 6-month-old leaves, and diseased 6-month-old leaves of P-122, a highly resistant clonal selection of Hevea brasiliensis Muell. Arg.

Water extract from 4-day-old leaves.--Light-brown to black pseudostromatic masses of cells grew on the surface and deep in the medium. Colonies appeared very flat, without any dumped stromatic masses. Conidial and spermogonial production was fair. Colonies grew to 13 mm in diameter, and 5 mm in height after 6 months.

No growth was observed on media prepared from alcohol extract of 4-day-old leaves (Table 6).

Water extract from healthy 6-month-old leaves.--Olive mycelium grew on the surface of the slant, with black stromatic mycelium in the center of the colony. The stromatic mycelium grew deep into the medium. Conidial production was good. Spermogonial production was good. Colonies grew to 7 mm in diameter, and 6 mm in height after 6 months.

Alcohol extract from healthy 6-month-old leaves.--Light-brown pseudostromatic mycelium grew on the surface and deep in the medium. No conidial or spermogonial production was observed. The one colony grew to 1 mm in diameter after 6 months.

Water extract from diseased 6-month-old P-122 leaves.--Olive and black mycelium were formed on the surface of the medium. Black stromatic tissue grew only on the surface of the slant. Conidial and spermogonial production was abundant, and droplets of spermatia oozed out of the spermogonia. Colonies grew to 7 mm in diameter within 6 months.

Alcohol extract from diseased 6-month-old leaves.--Light-brown to yellowish pseudostromatic tissue was developed on the surface of the medium. Fungal growth in this medium was scant and was not vigorous. No conidial or spermogonial production was observed. Colonies grew to 1 mm in diameter within 6 months (Table 6).

Media Containing Hevea benthamiana Leaf Extract

Water and 80 per cent ethyl alcohol leaf extracts were prepared from 6-month- and 2-month-old F 4542 leaves of an H. benthamiana Muell. Arg. clone.

TABLE 6

EFFECT OF ETHYL ALCOHOL AND WATER EXTRACTS ADDED TO MALT AGAR MEDIA

Extract Type	Spermogonial Formation[a]		Conidial Formation[a]		Av. Diameter of Colony (mm)	
	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
Hevea brasiliensis Muell. Arg.						
Diseased 36-day-old susceptible leaves	3	3	3	3	10	8
Dry 6-month-old leaves	-	-	-	-	-	-
Resistant F 232 6-day-old leaves	-	2	-	2	-	7
Highly resistant P-122 4-day-old leaves	-	2	-	4	-	13
Highly resistant P-122 6-month-old leaves	-	2	-	3	-	7
Diseased highly resistant P-122, 6-month-old leaves	-	2	-	2	-	7
Hevea benthamiana Muell. Arg.						
F 4542 2-month-old leaves	-	4	-	2	-	6
F 4542 6-month-old leaves	-	-	-	-	-	-
Control		1		1		1

[a]Arbitrary numerical rating for percentage of spermogonial and conidial formation: 4, above 75 per cent; 3, 50-75 per cent; 2, 25-50 per cent; 1, below 25 per cent; 0, none.

[b]Alcohol extract.

[c]Water extract.

Water extract media from 2-month-old leaves.--Black stromatic tissue was formed on the surface of the medium. The medium surrounding the stromatic tissue was reddish-black in contrast to the light-red color of the remainder of the medium. Deep in the medium, olivaceous mycelium formed large clumps of pseudostromatic tissue. Long thin hyphae were observed on the surface of the stromatic tissue. Conidial formation was moderate. Spermogonial formation was abundant. Colonies grew to 6 mm in diameter and 5 mm in height within 6 months (Figure 22).

Alcohol extract from 2-month-old leaves.--Light-brown to yellow clumps of mycelium grew on the surface and deep in the medium. No conidial or spermogonial production was observed. Colonies grew to 1 mm in diameter within 6 months.

Water extract from 6-month-old leaves.--Conidial seeded on this medium germinated readily but produced no growth (Table 6).

Alcohol extract from 6-month-old leaves.--Light-brown to yellow clumps of pseudostromatic mycelium were observed on the surface of the medium. No conidial or spermogonial production was observed. Colonies grew to 1 mm in diameter in 6 months.

Media Containing Rubber Latex

An experiment was conducted to determine the presence of a possible fungus growth source in Hevea latex.

Fubber latex Firestone S-4[1] (with an approximate rubber

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[1]Furnished through the courtesy of Dr. O. D. Cole of the Firestone Tire and Rubber Company, Akron, Ohio.

content of 61 per cent dry rubber, and 62.5 per cent solid content), preserved in 0.7 per cent ammonia was placed in a beaker inside a glass plate with 1N sulfuric acid to remove the ammonia preservative. After 2 days, when the ammonia preservative odor was almost gone, the latex was mixed with water agar in proportions which reduced the weight of dry rubber and solid content from 56.25 to 0.625 g per liter.

No growth was observed in any of the concentrations tested. The conidia germinated very slowly in the low concentrations while at higher concentrations, from 15.626 g to 56.25 g, no germination was observed. The conidia formed long germ tubes in the typical manner. At 2 and 4 weeks after seeding the conidia no further growth was observed in any of the concentrations.

Microchemical Studies

Microscopic difference observed between fungus invaded susceptible and resistant leaves was the abundant production of a yellow substance soon after fungus penetration of resistant leaf tissue and in old lesions of susceptible tissue. There was also observed the presence of sclerenchyma-type cells in lesions of old resistant leaf tissue. The chemical nature of the yellow material and of the sclerenchyma-type cells was studied with the aid of microchemical methods.

Microchemical tests for glucosides, hemicellulose and lignin were made with fresh and cleared leaf tissue.

Glucosides

The presence of saponins and tannins was studied following Johansen's (11) tests. Saponin-cleared sections of diseased resistant

leaflet tissue were placed on a slide with 2 drops of concentrated sulphuric acid. The sections changed from yellow to red, and ultimately violet. The sections were then placed for 24 hrs. in a barium hydroxide solution, washed in a weak aqueous solution of calcium chloride, and changed to a 10 per cent aqueous potassium bichromate. The yellow material in diseased young leaflet tissue gave no reaction and showed no change of color. The sclerenchyma cells gave a negative saponin test, as they remained brownish-red. Johansen (11) stated that tannin-containing cells became brownish-red during the reaction.

Tannin.--Fresh and cleared sections of fungus-invaded susceptible and resistant leaflet tissue were placed on a slide with a 10 per cent ferric chloride solution to detect the presence of tannins following Johansen (11). The yellow material present in young diseased resistant leaves near infection sites did not stain with ferric chloride. The sclerenchyma-type cells present in old diseased resistant leaflets turned blue-black with ferric chloride. No blue, blue-green, or black color was observed in lesions of young and old diseased susceptible leaflets. The yellow material observed near the old lesions gave a negative reaction with ferric chloride.

Hemicelluloses

Hemicelluloses are considered to be those compounds that yield pentosans, galactose or mannose upon hydrolysis. Johansen (11) stated that there are 2 kinds of hemicelluloses: (A) Those entering into the constitution of permanent cell walls, and (B) those occurring in storage organs.

The only hemicelluloses studied were those entering into the constitution of cell walls.

Xylan, Araban.--The phloroglucin-hydrochloric test was used to determine the presence of arabans and xylans. None of the fresh and cleared leaflet pieces gave the cherry-red color typical of xylose, arabinose.

Methyl Pentoses.--Fresh and cleared leaf sections of diseased susceptible and resistant leaflets were placed on a slide with 2 drops of acetone, 1 drop of concentrated hydrochloric acid was added, and the sections warmed gently for 15 minutes. Sections of susceptible leaflets gave negative results, as no cherry-red color was observed in the yellow material. Resistant leaflet sections gave a cherry-red color first and changed to dark brown-red later.

Lignin

Fresh and cleared sections of invaded susceptible and resistant leaflets were tested for the presence of lignin using an ammonical silver nitrate solution. None of the sections gave the typical black color of the reaction.

Microchemical test of the abundant yellow material found near lesions of young diseased resistant leaflets gave negative results. Sclerenchyma-type cells observed in pin-point lesions of old diseased resistant leaflets gave a positive test for tannins, and methyl pentoses.

Chromatographical Studies

Extracts were prepared from susceptible, resistant, healthy and diseased leaves of *H. brasiliensis* and healthy *H. benthamiana* leaves and were assigned numbers arbitrarily.

Chromatograms were prepared with quebrachitol, d-inositol, and i-inositol as references, and the prepared extracts spotted on No. 1 Whatman filter paper and developed in 4 solvent systems following Lederer and Lederer (16).

Solvent systems.--The best preparation of spots was obtained with the following solvent systems v/v: n-propanol-acetic acid-water (A), 7:1:2; n-propanol-acetic acid-water (B), 6:2:2; and phenol-water (C), 8:2. Spots were the sharpest with a n-butanol-ethanol-water (D), 10:1:2 solvent.

Dipping agents.--Various dipping agents have been used to detect the presence of sugars in chromatograms. Resing (23) used a strong ammoniacal silver nitrate to detect both sugars and alcohols. For sugars detection only he used aniline-trichloroacetate. Smith (27) in 1954 used the method described by Trevelyan (28), which is based on Feigl's test for reducing sugars. Bolle-Jones (3) used aniline phthalate and naphthoresorsinol in a hydrochloric phosphoric acid mixture as dipping agents.

The general treatment used for the detection of carbohydrates on the chromatograms was a modification of Trevelyan's reagents (28). The first reagent solution was prepared by diluting 0.1 ml of saturated aqueous silver nitrate solution to 20 ml of acetone. The aqueous silver nitrate was added dropwise with shaking, until the silver

nitrate which separates on addition of acetone had redissolved. Spreading of the spots was limited due to the sparing solubility of sugars in acetone (0.014 per cent at 23° C. for crystalline glucose). The second reagent, used also as a dipping agent instead of a spray, was prepared by dissolving 20 g of sodium hydroxide in 1 liter of 80 per cent ethanol (v/v). Upon dipping in this reagent, brown silver oxide was immediately produced.

Development procedure.--One dimensional chromatograms were prepared by placing a total of 5 ul (1 ul at a time, allowing the drops to dry, and repeating 5 times) along a line 2.5 cm from one end of a 20 cm long and 29 cm wide sheet of No. 1 Whatman filter paper. Ascending chromatograms were run in 1 gallon wide-mouth jars, covered with flat square pieces of glass, and vaseline on the rim of the bottles to form an air-tight seal.

The solvents A, B, C, and D were placed in a gallon jar and allowed to stand for 4 hours. Prepared chromatograms were rolled so that they would stand as a cylinder, clipped together at the uppermost edge with paper clips and placed in the jars so that the bottom edges of the cylinder would not touch. The chromatograms were allowed to run at room temperature (24° C) until the solvent fron reached a 1 cm distance from the top edge of the paper. The chromatograms were allowed to dry between dippings.

The chromatograms were first dipped continuously in the aqueous acetone solution for about 3 minutes. After allowing to dry, they were dipped in the ethanolic sodium hydroxide solution until the spots became dark brown, taken out and rinsed in tap water before the

background became light brown. Rinsing of the chromatograms in tap water was followed by dipping in a 50 per cent diluted solution of sodium thiosulfate. The sodium thiosulfate was washed off with running tap water and the chromatograms dried and stored in the dark.

Preparation of leaf extract.--The following procedure was found satisfactory for the preparation of leaf extracts suitable for both the chromatographic examination and quantitative estimation of the sugars present. Two g of fresh laminae were soxhlet extracted with 120 ml of 80 per cent ethyl alcohol for 2 hours. The extract was evaporated under reduced pressure at 30-40° to approximately 10 ml. This volume was partially clarified by filtering through diatomaceous earth (Celite), and made up to a volume of 50 ml with water. Twenty-five ml of this solution was concentrated under reduced pressure to a volume of 1 ml, and the remaining 25 ml were transferred to a screw-cap vial and stored at 0° C.

Inositol and Quebrachitol Determination

In early tests, it was determined that the quebrachitol used by Blazquez and Owen (2) in previous nutritional studies of Dothidella ulei P. Henn was not a pure compound, but that it contained large amounts of i-inositol and an unknown compound. It was suspected that because of the close R_f values of the unknown and i-inositol, the compound might have been the d- or l-enantiomorph of i-inositol.

The d-enantiomorph of l-inositol and a purified form of quebrachitol were obtained from the California Corporation for Biochemical

Research[1] in an attempt to identify the impurity found in the quebrachitol used previously. An additional purified form of quebrachitol was obtained from the Firestone Tire and Rubber Company.[2]

Solutions were prepared in concentrations of 4 mg per ml of the 3 quebrachitol compounds: impure quebrachitol, purified quebrachitol (California Corp.), purified quebrachitol (Firestone Co.); and of the d- and l- forms of inositol. One dimension chromatograms were run in solvents A, B, C, and D. In all the solvents tested, it appeared that the unknown impurity of the first quebrachitol used was d-inositol, as the R_f values of the impurity and d-inositol were the same.

Chromatographical determinations of leaf extracts.--Chromatograms prepared for the detection of possible differences between leaf extracts were run twice, with drying in between following Crossan and Lynch (4). Unidirectional chromatograms of leaf extracts from Hevea clones with varying degrees of resistance showed no significant difference in d- and l-inositol. The extracts from healthy and diseased, resistant and susceptible, showed the presence of both d- and l-inositol (Figure 23). The R_f values for d- and l-inositol are shown in Table 7. The presence of quebrachitol could not be determined as other sugars spots overlapped the area where the quebrachitol reference was found. The R_f values of quebrachitol are shown in Table 7.

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[1]California Corporation for Biochemical Research, 3625 Medford Street, Los Angeles 63, California. Courtesy of Dr. H. J. Teas.

[2]Courtesy of Dr. O. D. Cole. Firestone Tire and Rubber Company, Akron, Ohio.

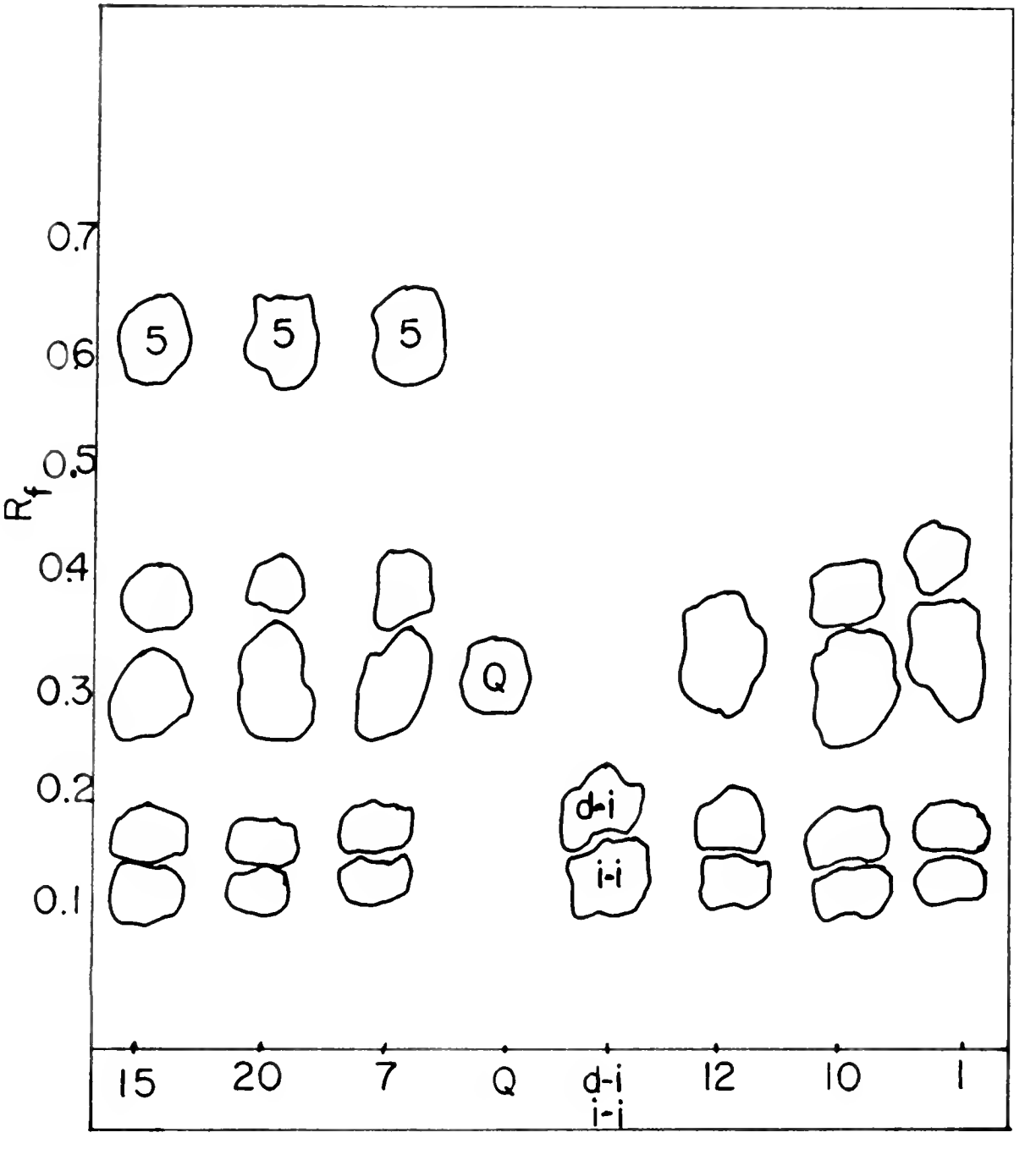

Figure 23. Chromatographic pattern of compound 5, which was present in extracts from healthy leaves (15, 20, and 7) but not on those from diseased leaves (12, 10, and 1). Known standards are of quebrachitol (Q), d- and l-inositol (d-i, i-i). Paper, Whatman No. 1; solvent, n-propanol-acetic acid-water, 6:2:2.

TABLE 7

R_f VALUES OF QUEBRACHITOL, INOSITOLS, AND COMPOUND 5

	Solvents		
	n-Butanol-Ethanol-Water, 10:1:2 (D)	n-Propanol-Acetic Acid-Water, 7:1:2 (A)	n-Propanol-Acetic Acid-Water, 6:2:2 (B)
Quebrachitol	0.11	0.33	0.41
d-inositol	0.07	0.19	0.23
i-inositol	0.03	0.14	0.16
Compound 5	0.30	0.65	0.65

In unsprayed chromatograms a yellow spot (compound 1) was detected in extracts of young leaves regardless of their degree of resistance (Table 8). Upon treatment with diazotized anisidine the yellow spot gave a pink color, and under ultra-violet light, long wave Black-ray B-100, it acquired a light absorbing dark-brown color (Figure 24).

Under ultra-violet light and ammonia fumes 3 blue fluorescent spots were observed. They were numbered 2, 3, and 4 in order of increasing R_f value. Table 8 shows the R_f value of the 3 fluorescent compounds. Extracts of young susceptible leaves showed the presence of the 3 compounds, while young resistant leaves did not. The 3 compounds did not react with diazotized anisidine or with aqueous silver nitrate in acetone (Figure 25).

Chromatograms prepared from extracts of diseased and healthy leaf extracts showed a spot at R_f, 0.6 to 0.7, present only in healthy leaf extracts. The spot (compound 5) did not fluoresce under ultra-violet light, and did not react with diazotized anisidine. Upon treatment with the silver-acetone reagent, the spot reacted with the silver to give a dark-brown spot. R_f values for compound 5 are given in Table 7. Trevelyan (28) reported that reducing sugars will reduce silver to silver oxide to form a dark-brown spot. It is possible that compound 5 may be a reducing sugar.

TABLE 8

R_f VALUES OF FLUORESCENT COMPOUNDS FOUND IN EXTRACTS FROM LEAVES OF HEVEA CLONES[1]

	Solvents			
	Phenol and Water 8:2 (C)	n-Butanol-Ethanol-Water, 10:1:2 (E)	n-Propanol-Acetic Acid-Water, 7:1:2 (A)	n-Propanol-Acetic Acid-Water, 6:2:2 (B)
Brown compound 1	0.35	0.45	0.55	0.55
Blue compound 2	0.60	0.50	0.60	0.70
Blue compound 3	0.70	0.60	0.70	0.80
Blue compound 4	0.80	0.70	0.80	0.80

[1] R_f ratio of distance traveled by spot to distance traveled by solvent front.

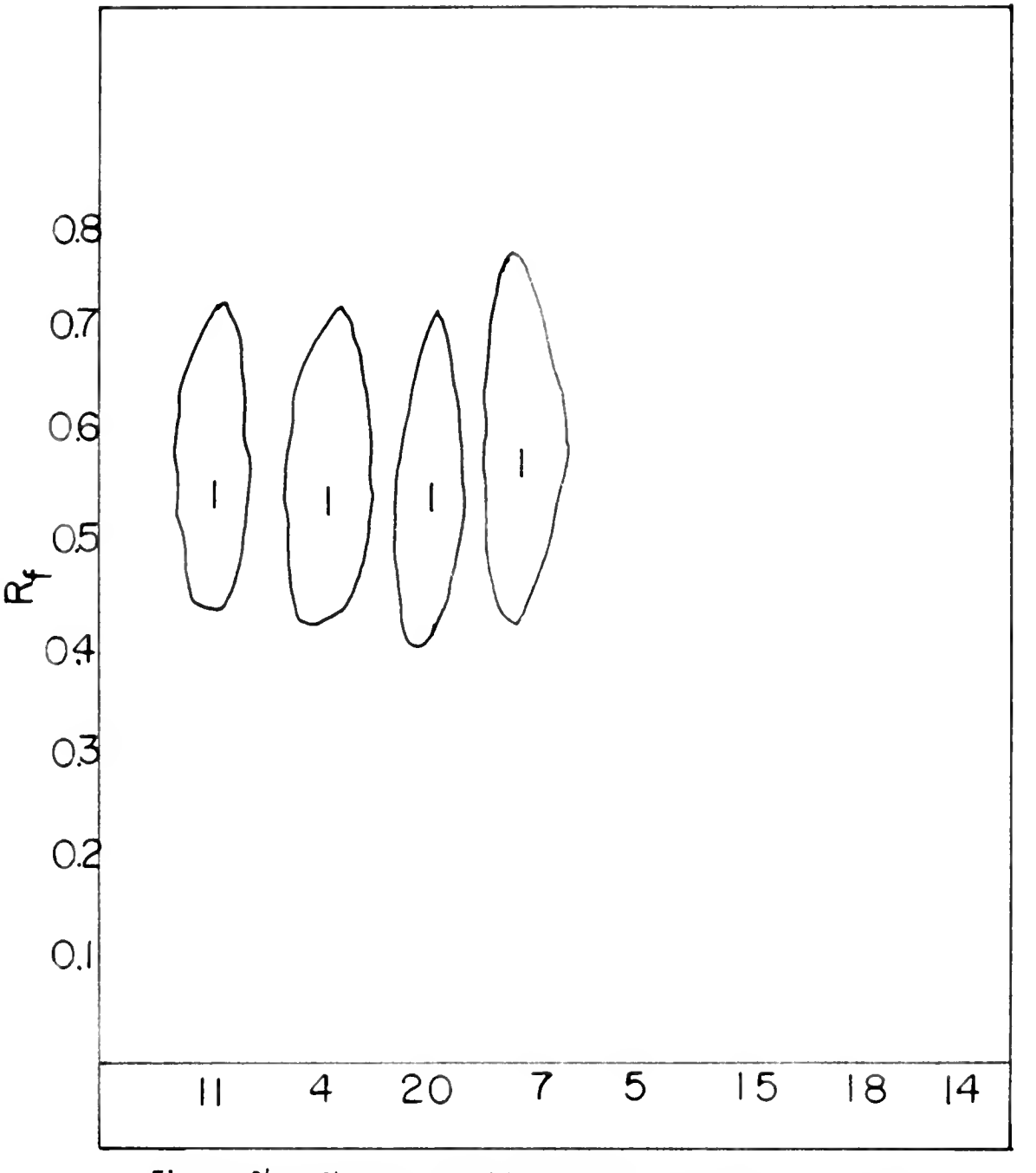

Figure 24. Chromatographic pattern of yellow compound 1 present in extracts from young leaves (11, 4, 20, and 7), but not in those from old leaves (5, 15, 18, and 14). Paper, Whatman No. 1; solvent, n-propanol-acetic acid-water, 6:2:2.

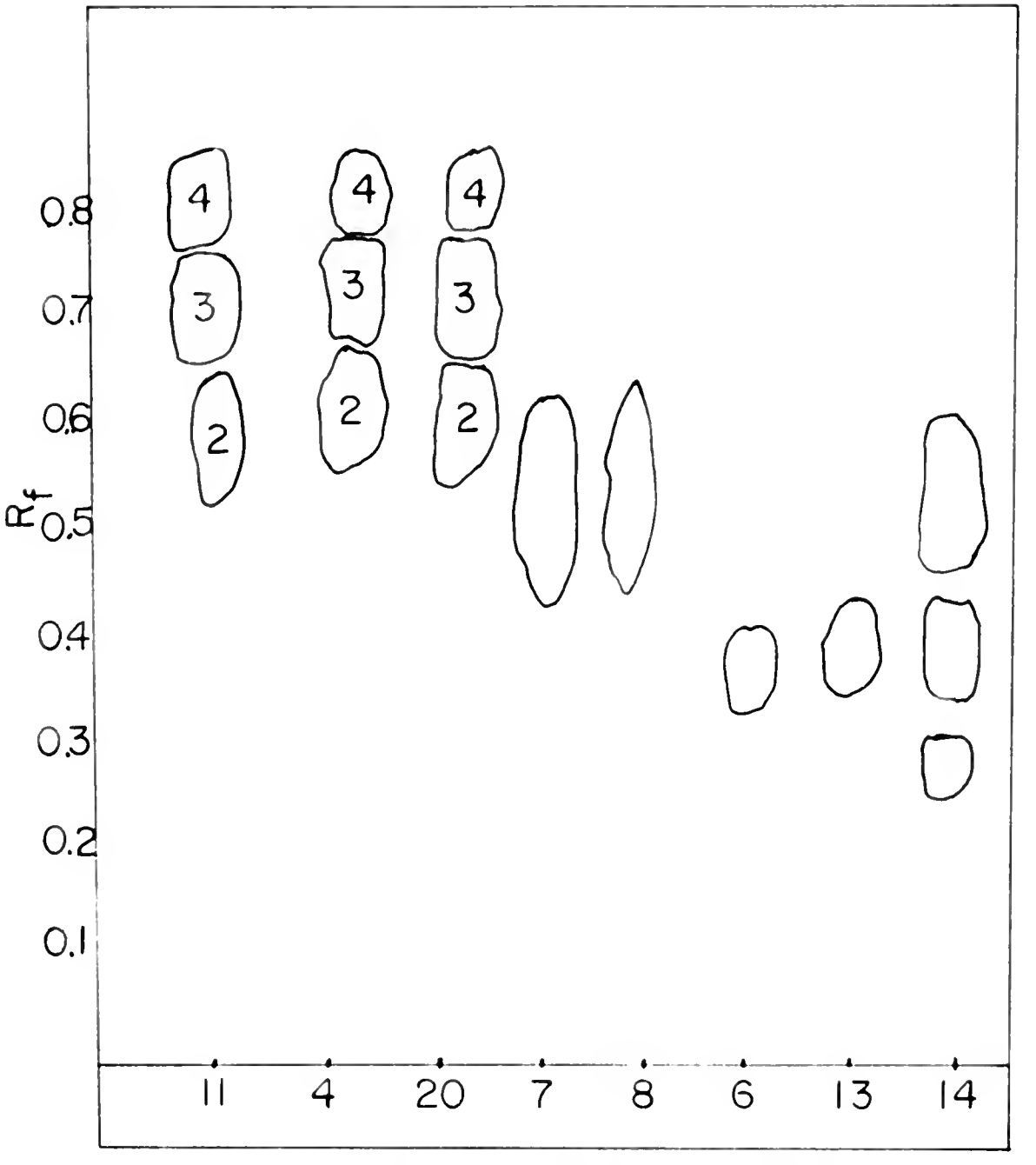

Figure 25. Chromatographic pattern of fluorescent compounds (2, 3, and 4) present in extracts from young susceptible leaves (11, 4, and 20), but not in those from young resistant leaves (7, 8, 6, 13, and 14). Paper, Whatman No. 1; solvent, n-propanol-acetic acid-water, 6:2:2.

DISCUSSION

Inoculation experiments carried out with 4 resistant clones of Hevea rubber did not agree with Langford's work (15). Under 100 per cent humidity it was possible to observe the rapid symptom expression of susceptible as well as resistant clones. In highly resistant leaflets (Langford's immune) small dot-like lesions were very conspicuous 3 days after inoculation. The normal reddish-brown color changed to yellow in areas bordering the lesions. Langford (15) stated that lesions on resistant and highly resistant leaves were smaller than lesions on susceptible leaves, appeared much later, and caused less extensive damage. It is probable that he might have observed the development of lesions at less frequent intervals missing the similarity on young resistant leaves, in which case his findings would agree with the results obtained herein.

Lesions on resistant leaflets were similar to the susceptible ones the first 24 hours after inoculation, after which discoloration of marginal areas and some distortion occurred. These observations did not agree with Langford's findings (15).

Hypertrophy of the center of the lesions, and the initiation of sporulation was observed in susceptible leaflets, while the lesions on resistant leaflets showed neither.

Very young leaflets of resistant and highly resistant clones developed normally when heavily inoculated but were shed after 3 weeks. They remained on the tree longer than the young leaflets of

susceptible clones. The resistant and highly resistant leaflets were capable of inhibiting the growth of the fungus, and were able to grow normally although heavily infected. The resistance may be due to a hypersensitivity of the cells to fungus invasion. Attacked cells became devoid of chlorophyll and gradually collapsed. Cells bordering the lesions became disorganized and appeared to be filled with a yellow granular material.

Microchemical tests indicated that the nature of the yellow material was not related to lignin or tannin, as tests for both were negative.

It is believed that extraction of the yellow material and identification of its chemical composition might be accomplished by extraction with selected solvents, and testing for methyl pentoses, pentosans, and hemicelluloses.

Resistant leaves might produce or contain a substance which could be changed to this yellow material by fungus enzymes soon after penetration. In susceptible leaves the formation of the yellow material occurred later and possibly required a higher concentration of fungus enzyme. In susceptible leaves this yellow accumulation occurred only in lesions of old leaves.

Certain factors may be involved in the phenomena of fungal growth inhibition in the resistant and highly resistant leaves. Host reaction may be a possible explanation, whereby a substance might undergo a structural change when attacked by enzymes produced by the fungus. The changed substance may be toxic to the fungus or

to the host, preventing any further development of infection because of supersensitivity of the host or as inhibiting growth of the fungus.

It is quite possible that the yellow material may not be growth inhibiting, but rather a waste product which merely accumulated in the tissues wherever fungus mycelium penetrated. Growth inhibition in this case may be due to the absence of a substance required for growth of the fungus. It is also possible that growth inhibition in resistant leaves may be due to the presence of a substance in larger amounts than required; however on susceptible leaves this substance may be present in smaller quantities which would allow growth of the fungus.

Cross sections of highly resistant leaflets 6 months after inoculation showed no collapsed cells or necrotic areas. The only signs of fungus invasion were groups of sclerids distributed near points of infection. The presence of these sclerids may be parallel to the formation of brachysclerids as reported by Foster (7). Tissue that collapsed after infection might have left definite gaps in the leaf structure. These gaps might have been filled by parenchyma cells which divided to varying degrees and were transformed into brachysclerids, thus "repairing" the broken leaf structure. Brachysclerids are often found near wound tissue according to Foster (7). The sclerids found throughout the diseased resistant leaf tissue gave a positive tannin test with ferric chloride.

Growth of the fungus was inhibited in media prepared from

alcohol extracts of diseased highly resistant leaves, but was favored in media from the alcohol extract of diseased susceptible leaves. Media prepared from water extracts of highly resistant and susceptible, young and old leaves, greatly favored growth of the fungus. This suggested that a growth inhibitory substance could be extracted with ethyl alcohol but not with water. This substance may either be the product of fungus enzyme action on host cell contents or a secretion of the host tissue as a protective effort when irritated by the toxin produced by the invasion of the fungus. It was believed that an alcohol soluble substance which inhibited growth of the fungus had been broken down by fungus enzymes in diseased susceptible leaves. Thus it would allow fungus growth in media from alcohol extracts of diseased susceptible leaves.

Media prepared from water extracts would allow growth of the fungus due to the absence of the inhibitory substance.

Similarly it might be said that if the water soluble substance were required for growth of the fungus, then it might be possible that media prepared from water extracts would have sufficient amounts of the substance and would favor fungus growth. Alcohol extracts, on the other hand, would lack the water soluble substance and would not favor fungus growth. Growth on alcohol extract media from diseased susceptible leaves may have been due to fungus enzymatic breakdown of the water soluble compounds into an alcohol soluble form that would favor fungal growth.

A yellow spot (compound I) was found in chromatograms from

extracts of healthy young leaves regardless of their degree of resistance. It is believed that this compound 1 may be of a steroid nature, and not unlike the yellow compound found by Kuc et al. (13), and later identified as chlorogenic acid.

Some of the differences could be attributed to substance only present during each stage of leaf development, but distinct differences were detected between the resistant and susceptible leaf extract under ultra-violet light. It is believed that additional work should be carried out with the ultra-violet fluorescent compounds 2, 3, and 4, as they may be found to be of some importance in the study of parasitism.

It is quite possible that many of these differences between the young and old leaflets, between resistant and susceptible clones, and between health and disease, may or may not be involved in the phenomena of resistance. They are important and need further consideration since they may help clarify the complicated biochemical host-parasite relationship involved in the host resistance to D. ulei.

Two solvents were used for leaf extraction, as a consequence it should be pointed out that results obtained should be compared with results obtained from extracts by other organic solvents.

It is felt that extracts from leaves should be prepared with various solvents and added to natural media to test fungus growth stimulation or inhibition. Thus it would be possible after

extensive experimentation to formulate a hypothesis dealing with the nature of the host-parasite relationships between Dothidella ulei and the Hevea rubber tree.

SUMMARY

Related factors involved in the infection and growth of the fungus Dothidella ulei P. Henn. were studied on various leaf stages of the Hevea rubber tree. The 6 leaf stages were set up arbitrarily, depending upon growth characteristics of the Hevea rubber tree.

A black exudate was produced on diseased susceptible and resistant Stage I leaves.

Results of inoculation experiments generally agreed with those previously described. Microscopic observations were made on cleared leaf and paraffin embedded sections of diseased susceptible, resistant and highly resistant leaves. Distinct differences were detected between resistant and susceptible leaves. Appressoria were abundantly produced by germinating conidia on resistant leaves, while only a few were observed on susceptible leaves. Direct penetration was usually observed on susceptible leaves.

A yellow material was detected soon after fungus penetration of resistant leaf tissue and in old lesions of susceptible leaves. The yellow material gave negative results with various microchemical tests.

Sclerenchyma-type cells were observed in lesions of old resistant leaves, these cells gave a positive tannin, and methyl pentose test.

In resistant and highly resistant leaves the fungus penetrated the epidermis, but no further development was observed although

young resistant and highly resistant leaves showed cell disorganization and tissue collapse when diseased. No sporulation, stromatic mycelium, or fungus fructification were detected in any of the cleared leaf of paraffin embedded sections.

Chromatographical determinations showed small amounts of d- and i-inositol present in the quebrachitol previously used in culture media. Cultural studies using chemically pure quebrachitol showed no significant differences between the contaminated and the pure quebrachitol.

There was no significant differences between 8 sugars and 1 sugar alcohol as sources of carbon tested in basal semi-synthetic media. Best growth was obtained in media using the vitamin-amino acid combination i-inositol and l glutamic acid.

Media prepared from water extracts of Hevea brasiliensis clones gave good growth of the fungus. No growth was obtained in media from 6-month-old dried leaf water extract, or from Hevea benthamiana (F 4542) 6-month-old leaves.

No growth was obtained in media prepared from alcohol extracts. The only exception was the diseased 36-day-old susceptible leaf extract.

In chromatographical determinations of susceptible, resistant, and highly resistant alcohol leaf extracts, it was possible to detect the presence of d- and i-inositol. The presence of quebrachitol, 2 mono-methyl ether of l-inositol, could not be detected.

In chromatograms of healthy and diseased, resistant and susceptible leaf extracts, a yellow spot (compound I) with an R_f value

of 0.42 was detected in undeveloped chromatograms. Under ultra-violet light and ammonia fumes 3 blue fluorescent spots (compounds 2, 3, and 4) were detected. The R_f values in solvent A were 0.6, 0.7, and 0.8 respectively.

Compound 1 changed to a brown absorbent color when illuminated with ultra-violet light.

Compound 1 was found in all extracts from young leaves, while compounds 2, 3, and 4 were found only in extracts from young susceptible leaves and were not detected in extracts of young resistant leaves.

Compound 5 was found in extracts prepared from healthy leaves, but not in those from diseased leaves, and when treated with the silver-acetone developer, it reacted to give a brown spot.

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BIOGRAPHICAL DATA

Carlos H. Blazquez was born October 9, 1926, at Mexico City, Mexico. He attended elementary school in that city, and graduated from John Marshall High School in Los Angeles, California, in February, 1951. He received an Associate of Arts degree from Chaffey College in Ontario, California, in June, 1952, a Bachelor of Science degree from the University of California at Davis, California, in June, 1955, and a Master of Science degree from the University of Florida, Gainesville, in January, 1957. He began work on the doctor's degree at the University of Florida in February of 1957, where he held a graduate fellowship from the Firestone Tire and Rubber Company, and completed the degree in June, 1959.

He was called to active duty in December, 1944, and served two years with the U. S. Naval Reserve. He also served with the U. S. Coast Guard from August, 1946, until August, 1950.

He worked during the summers of 1954 and 1955 as an Inspector of Plant Pathology and as a Junior Plant Pathologist for the Bureau of Plant Pathology, California State Department of Agriculture. He is a member of the American Phytopathological Society, the American Society of Plant Physiologists, the Mycological Society of America, and the Phi Sigma Honorary Biological Society.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 8, 1959

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